EARLY STEPS IN NEURAL INDUCTION

Matthew James Stower

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I Matthew James Stower confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

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Neural induction is the instructive interaction whereby signals emitted from the organizer direct cells in the ectoderm to a neural fate and thereby to form the neural plate. Recent work in many labs has suggested that it involves a hierarchy of molecular events. Here, I investigate the earliest steps in the neural induction cascade and the signals that define them.

Many genes expressed during the neural induction cascade have been shown to be regulated by FGF. However the signals that induce three of the genes, *Bert*, *TrkC* and *Obelix*, are unknown. I therefore tested candidate signalling molecules by misexpression analysis. While *Obelix* is also regulated by FGF, none of many factors tested, including FGF, retinoic acid, somatostatin, noggin, insulin, and increasing intracellular calcium were able to induce expression of *TrkC* or *Bert*.

BMP also plays an important role in neural induction, I therefore studied how cells may integrate TGFβ signalling through Smad1 and Smad2. I used a BiFCo approach to investigate Smad protein binding interactions in culture and in vivo, however this did not turn out to be a useful method for this question.

Finally I investigated the ground-state of the epiblast at the start of the neural induction cascade. Culture of early epiblast explants showed, unexpectedly, that cells initially enter a state similar to that of the neural plate border, confirmed by their subsequent differentiation into lens. This correlates with the finding that BMP signalling in vivo only affects cells of the neural plate border region and suggests why explants can be neuralized by BMP.

Overall, the experiments reveal a hitherto unknown importance of a neural border cell-state, and suggest that lens is the ground state at the start of the neural induction cascade.

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Chapter One

General Introduction

Gastrulation

Gastrulation is the process by which the three definitive germs layers; endoderm, mesoderm, and ectoderm are formed from a single layered sheet of cells, the blastoderm. The blastoderm can be considered a multipotent progenitor pool from which all the cells of the embryo derive. Gastrulation therefore marks the fundamental separation of fates in these cells. Cells differentiating in each of the germ cells layers become progressively restricted in the specific fates that they can give rise to. Thus, endoderm cells give rise to the digestive system, mesoderm cells gives rise to the muscles, skeleton, circulatory system, and the internal organs, and the outer most layer, the ectoderm, gives rise to the dermis (skin) and the nervous system (central and peripheral).

Although, there are large differences in the size and morphology of blastula stage embryos between vertebrate classes, the process of gastrulation shares fundamental similarities including the large cell movements that will ultimately lead the endodermal and mesodermal progenitors leaving the ectodermal cells in the blastodermal sheet. The mesendodermal cells achieve this either by ingressing through a primitive streak structure in amniotes (such as mouse and chick) or inwards through forming a circular blastoporal structure in amphibia, or around the dorsal shield in teleosts.

Gastrulation in the Chick

At the time of laying, the stage-X (Eyal-Giladi and Kochav, 1976) chick blastoderm is a single-cell layered flat disc, with an inner area pellicida epiblast region whose cells gives rise to all the germ layers (Hatada and Stern, 1994), and an outer ring of cells, the area opaca epiblast which has

extra-embryonic fate. Between these cell regions is the marginal zone, a narrow region of cells, which are transcriptionally, but not morphologically distinguishable from the other cells of the epiblast (Seleiro et al., 1996; Shah et al., 1997; Skromne and Stern, 2001). There is also an anterior-posterior asymmetry at this stage, with a crescent shaped ridge of cells, termed Koller's sickle (Koller, 1882), at the margin of the area opaca that defines the posterior of embryo. The epiblast is also covered by two types of cell layer; the area opaca epiblast is covered by several layers of yolky cells with those that are closest to the epiblast layer firmly attached to it, these are termed the germ wall (Stern and Ireland, 1981). The area pellucida epiblast layer at this stage is also covered, by small clusters of cells formed from the shedding of cells from the area pellucida during laying (Kochav et al., 1980). These clusters of cells are the progenitors of an extraembryonic cell layer, the hypoblast, a region equivalent to the anterior visceral endoderm (AVE) in the mouse, which forms as the islands of cells spread and coalesce from posterior to anterior in the embryo, so that by stage-XIII (Eyal-Giladi and Kochav, 1976) a continuous sheet of cells completely covers the epiblast (Vakaet, 1970; Stern, 1990).

After this stage, the posterior germ wall margin cells and their progeny form another cell layer that moves anteriorly displacing the hypoblast from the posterior of the embryo, this event defines the stage-IV embryo (Eyal-Giladi and Kochav, 1976) and initiates the onset of gastrulation (Bertocchini and Stern, 2002). The endoderm and mesodermal progenitors in the area opaca epiblast now start to ingress at this posterior region marking the start of gastrulation; initially the ingressing cells form a triangular thickening of the epiblast (stage 1-2 (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976), which subsequently narrows and extends to the middle of the area opaca until it forms the full primitive streak at the midline of the embryo. At the anterior tip of the streak is a morphological node-like structure, Hensen's node, which is visible from stage 3^{+} (Hamburger and Hamilton, 1951). This node structure abuts the region of the epiblast, which will form the neural plate, a thickening of the ectodermal layer whose cells will give rise to the central nervous system. The cells that surround the neural plate in the surface ectoderm will give rise to the epidermal (skin) cells, and cells at the boundary

between the neural plate and epidermis, the neural crest and placodes, will give rise to the peripheral nervous system.

Thus, by the end of gastrulation the single cell layer of the blastula has given rise to the three cell-layered gastrula, and the cells that will form the nervous system are situated anteriorly in the surface ectoderm. The cellular sheet of cells in the neural plate progressively extends and flattens during development before folding in upon itself to form the neural tube, whose cranial region and trunk regions, respectively, will give rise to the brain and spinal chord of the central nervous system (CNS).

Induction & The Spemann-Mangold Organizer

The concept of Induction "the interaction between one inducing tissue and another responding tissue as a result of which the responding tissue undergoes a change in its direction of differentiation" (Gurdon, 1987) was an important advance in our understanding of how organisms develop. The first experiments that unveiled this developmental phenomenon suggested that the optic lobe of the brain induces the overlying ectoderm to form the lens of the eye (Spemann, 1901b; Lewis, 1904). This established the idea that development is regulative and not self-deterministic; regions of the embryo (clusters of cells) can have instructive roles that direct the fate of surrounding tissues.

Conceptually, induction provides a developmental system with a mechanism for coordinating both temporally, and spatially, the development of distinct cell types and tissues. It was Spemann and Mangold's pioneering experiments (Spemann and Mangold, 1924) that first identified that an inductive interaction is one of the earliest steps in the formation of the nervous system. The key experiment was a result of a series of transplantation studies where Spemann explored the nature of tissue interactions in early development. Spemann first transplanted pieces of ectoderm or neural plate of the same or different species of newt embryos into a heterologous position of a host embryo. These

pieces of tissue developed according to their new position (Spemann, 1918; Spemann, 1921). However, when he transplanted the dorsal lip of the blastopore of an early gastrula-stage newt embryo into a region fated to become epidermis, it did not develop according to its new position, but continued to develop according to its region of origin forming a secondary axis complete with neural tube, notochord and somites (Spemann, 1918; Spemann, 1921).

Initially Spemann interpreted this as solely due to self-differentiation of the grafted dorsal-lip tissue. However, in a subsequent experiment Spemann rotated the animal half of the gastrula embryo 90 degrees with respect to its vegetal half, and observed that the lower vegetal half containing the lip of the blastopore influenced the development of the overlying animal piece (Spemann, 1918). Spemann now asked whether the formation of the secondary axis in the transplantation experiment were due to the self-differentiation properties of the dorsal-lip or its influence on the surrounding tissue. The key experiment, carried out by Spemann's student Hilde Mangold, included a vital technical advance; a tissue transplantation technique that enabled them to distinguish between the cells of two species of newt. Thus, by transplanting the dorsal lip of the blastopore of *Triturus cristatus* embryo to the ventral ectoderm of a *Triturus taeniatus* host embryo they could identify the contribution of cells to the resultant secondary axis by distinguishing between the lightly pigmented *T. cristatus* and heavily pigmented *T. taenitus* cells. This lineage study showed that the main contributions of the grafted dorsal lip tissue were to the notochord, somites and floor plate, but the nervous system was almost entirely derived from the host cells (Spemann and Mangold, 1924).

This experiment clearly showed that an instructive interaction had changed the fate of cells of the hosts ventral ectoderm to become neural tissue instead of their normal fate, epidermis (Spemann and Mangold, 1924). Furthermore, the induced tissue in the second axis was patterned both antero-posteriorly and dorso-ventrally. Given this ability to induce and organize, Spemann termed the dorsal lip of the blastopore "The Organizer" (now referred to as

Spemann's- or The Spemann-Mangold Organizer). Whilst this embryonic induction is an experimental paradigm, it suggested that during normal development there is an instructive interaction that directs cells to a neural fate, termed neural induction. It prompted other researchers to ask whether cells with equivalent ability exist in other species, whether in short, neural induction is a general strategy of vertebrate development.

Identification of Vertebrate Organizers

In the years following the discovery of the organizer (Spemann and Mangold, 1924) transplantation experiments and lineage studies enabled researchers to identify regions that were functionally equivalent in other vertebrate species. For example, in avian embryos (chick, duck, and quail) transplantation of the tip of the primitive streak, Hensen's node, to either the prospective epidermis or to an extra-embryonic region induced a secondary axis (Waddington, 1930; Waddington, 1932; Waddington, 1936; Waddington, 1937). Similarly, the shield of teleosts (Oppenheimer, 1936a) and the mammalian node (mouse: (Waddington, 1934; Waddington, 1936; Waddington, 1937; Beddington, 1994)) are also able to induce a neural plate when transplanted to ectopic positions.

Remarkably, these vertebrate organizers are also able to induce when transplanted across classes (Waddington, 1934; Oppenheimer, 1936b; Kintner and Dodd, 1991; Blum et al., 1992; Hatta and Takahashi, 1996). For example, transplanted mammalian nodes (rabbit, mouse) can induce neural tissue in a chick (Waddington, 1936; Waddington, 1937; Zhu et al., 1999) or *Xenopus* host (Blum et al., 1992) and a transplanted Hensen's node from a chick embryo can induce neural tissue when transplanted into rabbit (Waddington, 1934), *Xenopus* (Kintner and Dodd, 1991) or zebrafish (Hatta and Takahashi, 1996) embryos. Collectively, these studies strongly suggest that in vertebrates neural fate in the developing embryo is specified by an inductive process, and the mechanisms of neural induction; both the inductive signals and response of induce tissue, have been conserved throughout vertebrate evolution.

Ever since classical grafting experiments identified the organizer, investigations focused mainly in a handful of vertebrate model organisms and increasingly experiments from stem cell culture studies, have begun to unravel the conserved events of neural fate specification. These events can be divided into two areas: 1) the signalling molecules capable of non-cell autonomous control of neural cell fate and, 2) the reciprocal interaction of the induced tissue acquiring a neural fate. However, although some of the signalling molecules and events within prospective neural cells have been identified, there is still much we don't understand, and the emerging complexity gained from different experimental systems, each with distinct experimental approaches has meant an integrated model of neural fate acquisition has been difficult to reach.

Neural Induction Signals

The Search for the Neural Induction Signal.

The search for "the neural inducer" (also called the "evocator" in early studies), a proposed signal secreted from organizer tissue, began by testing the ability of substances to induce neural tissue in the newt embryo, the model where the organizer and neural induction had first been described (Spemann and Mangold, 1924). However, it soon became apparent that many heterologous substances (substances without common structure or activity) were able to induce ectopic neural tissue when grafted into gastrula stage newt embryos including, fatty acids, ribonucleoproteins and even sand particles (SiO_2) (Holtfreter, 1951; Holtfreter and Hamburger, 1955) Furthermore, chemical substances which clearly could not be the signal such as methylene blue were also able to neuralise pieces of ectoderm (Holtfreter and Hamburger, 1955).

In short, the search for the neural inducer in the newt was stymied by the following problems; 1) If many substances can induce ectopic neural tissue, which is the correct one? 2) Mechanical interference of the tissue by a foreign

substances can interfere with cell movements leading to self-regulation to form ectopic structures. 3) Even if a substance is introduced into a newt gastrula and found to cause neuralisation, it cannot be taken as evidence that the substance is the same as, or related to the endogenous signal. One of the upshots of this period of inertia and confusion in the field is that it coincided with the development of another amphibian *Xenopus laevis,* by Peter Nieuwkoop, as a model organism. It was the use of this model species combined with the advances in molecular biology that finally led to the first break-through in our understanding of the signals involved in neural induction, nearly seven decades after the Spemann-Mangold experiments.

BMPs & The Default Model of Neural Induction

The first explicit molecular model attempting to explain neural induction emerged from experiments in *Xenopus* that put Bone Morphogenetic Proteins at the centre of the neural fate decision. It was known that isolating and culturing ectodermal tissue from *Xenopus* gastrula stage animal caps would give rise to epidermal tissue, this assay had therefore been used as a test bed to identify proteins that could induce mesoderm (Nieuwkoop, 1969). However, if the animal cap tissue was instead dissociated into single cells by immersion in media depleted of calcium and magnesium for a short time (~ 3 hours) prior to reaggregation, the cells now gave rise to neural tissue (Born et al., 1989; Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989; Saint-Jeannet et al., 1990).

Why was there change of fate in the animal cap cells after dissociation? One interpretation was that a molecule inhibiting neural fate was being diluted by the dissociation procedure (Hemmati-Brivanlou and Melton, 1994). The nature of the hypothesized inhibitory molecule was first suggested by the discovery that injection of a dominant-negative receptor of activin, a (TGF-β) Transforming Growth Factor β-related factor, could convert animal caps to neural tissue with out dissociation (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994). Subsequently, several genes with neuralising activity, including Noggin (Smith and Harland, 1992; Lamb et al.,

1993; Smith et al., 1993) Chordin (Sasai et al., 1994; Sasai et al., 1995) and Follistatin (Hemmati-Brivanlou et al., 1994), were found to be expressed by the organizer and shown to be able to bind and inhibit TGF-β, Bone Morphogenetic Protein (BMP) signalling (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). Furthermore, BMP4 was shown to be able to inhibit neural fate while promoting epidermal differentiation in animal cap assays (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Together, these observations led to the "Default" or "Ground-State" model of neural induction (Hemmati-Brivanlou and Melton, 1997a) which reinterpreted the role of the Organizer. The model proposed that cells in the ectoderm have a neural "ground-state" or "autonomous state of differentiation" (Hemmati-Brivanlou and Melton, 1997a), which is inhibited by BMPs. Thus, rather than providing a stimulating or directional signal, the role of the organizer in the Default model is to protect cells in the ectoderm from BMP signals, thereby allowing them to differentiate into their default, neural fate (Hemmati-Brivanlou and Melton, 1997a; Levine and Brivanlou, 2007). In support of this model, BMP4 is initially expressed broadly in *Xenopus* ectoderm but subsequently lost from the future neural tissue when the organizer forms (Fainsod et al., 1994). Also, by experimentally inhibiting the action of BMP signalling through injection of either dominant-negative BMP receptors (Thomsen and Melton, 1993; Graff et al., 1994; Suzuki et al., 1994) non-functional forms of BMP proteins (Hawley et al., 1995), anti-sense morpholinos against *Bmp4* (Sasai et al., 1995), animal caps cut from these embryos will develop into neural tissue. Moreover, both BMP inhibitors, Noggin and Chordin are able to neuralise cultured animal caps (Lamb et al., 1993; Sasai et al., 1995).

However, because single or double mouse knockouts of the BMP antagonists Chordin and Noggin still have a nervous system (although they lack the most anterior structures) (McMahon et al., 1998; Bachiller et al., 2000; Belo et al., 2000; Mukhopadhyay et al., 2001) it had been thought that *in vivo*, BMP-inhibition alone is not sufficient for neural induction. In fact, an array of BMP antagonists have been identified that are either expressed in the organizer or nearby including *Cerberus* (Boumeester et al., 1996; Belo et al., 1997),

Gremlin (Hsu et al., 1998; Khokha et al., 2003), *Dan/Drm* (Pearce et al., 1999; Dionne et al., 2001; Eimon et al., 2001) and *Ogon/Sizzled* (Wagner & Mullins, 2002: Yabe et al., 2003). This redundancy of function may explain why single knockouts have little effect on neural development. Indeed, a triple morpholino mediated knockdown of three BMP antagonists (Chordin, Noggin and Follistatin) together in *Xenopus* results in an almost a complete loss of the neural plate (Khokha et al., 2005). Conversely, the depletion of three BMP signalling proteins (2,4,7) has the opposite effect – massive brain formation (Reversade et al. 2005). Moreover, if a quadruple knockdown is carried out, this time by injecting a morpholino against anti-dorsalizing morphogenetic protein ADMP (another member of the TGFB family) in addition to Bmp2, 4 and 7, then an even larger brain develops (Reversade et al., 2005). Together there is a large body of evidence for the role of BMP-inhibition in neural induction and for an inhibitory effect on neural tissue of the BMP pathway. However, there has been much debate about whether BMP inhibitors are the sole neural inducing signals of the Organizer and whether a "default" fate exists. Evidence mainly from experiments in the chick model began to challenge this one-pathway model.

The Challenge to the Default Model

The default model states that inhibition of BMP signalling alone is sufficient for neural induction (Hemmati-Brivanlou and Melton, 1997a). However, several observations in the chick began to contradict the model. Firstly, unlike in *Xenopus*, the expression pattern of BMPs (4 and 7) and BMP-inhibitors, including Noggin and Chordin, do not fit as predicted by the model which suggests that neural markers should be only expressed when BMP signalling is cleared from the tissue (Streit et al., 1998; Streit and Stern, 1999). Secondly, misexpression of BMP antagonists in the inner-third of the area opaca – an extraembryonic region of the embryo which is can respond to signals from a grafted node (a property known as 'competence'), does not lead to the expression of any known neural markers or neural plate-like columnar morphology in competent tissue (Streit et al., 1998; Streit and Stern, 1999). Furthermore, the formation of the endogenous neural plate is only

slightly narrowed, but not inhibited, when a source of BMP4 is introduced (Streit and Stern, 1999). Indeed, only the border of the neural plate, which gives rise to the neural crest and anteriorly the sensory placodes, is affected by BMPs (Streit and Stern, 1999). Addition of BMPs will shift the expression of border markers inwards towards the midline, whilst inhibition of BMP will shift the border territory outwards (Streit and Stern, 1999).

However, whilst these results suggest that inhibition of BMP signalling alone is not sufficient for neural induction in the chick, there is evidence for a role in the maintenance of neural fate: BMP4 transcripts and the level of the phosphorylated (activated) SMAD-1, the intracellular BMP signalling effector, is reduced in the forming neural plate but only after the expression of early neural plate markers (*Sox2* and *Sox3*) (Streit and Stern, 1999; Faure et al., 2002). Also, if a node is grafted then removed so that competent epiblast tissue is exposed to signals for only 5 hours (13 hours are required for induction of neural tissue (Gallera and Ivanov, 1964; Gallera, 1971a), BMP inhibition by Chordin is then able to stabilise the expression of the early neural marker *Sox3* (Streit et al., 1998). Similarly, overexpression of BMP4 in the forming neural plate can inhibit expression of the later neural marker *Sox2*, but the early neural marker *Sox3*, is not affected (Linker and Stern, 2004). Thus, these results suggest that whilst BMP antagonism plays a role in neural induction *in vivo* in the chick: 1) a single step of BMP-inhibition alone cannot replicate the effect of a grafted organizer in the non-neural ectoderm, 2) there are additional signalling pathways involved, and 3) that a signalling step(s) precede the requirement of BMP antagonism.

A Role for FGF signalling in Neural Induction

Experiments in the chick have suggested that although inhibition of BMP signalling is involved in neural fate acquisition, alone BMP inhibition is insufficient to induce a neural fate suggesting that other signals may be involved. A signalling pathway that had previously been implicated in neural induction is the Fibroblast Growth Factor (FGF) pathway, as animal caps cut from *Xenopus* embryos where FGF signalling had been blocked by

microinjection of dnFGFR1, a dominant negative FGF receptor, can no longer be neuralised by Noggin (Launay et al., 1996) or Chordin (Sasai et al., 1996) treatment. Furthermore, studies using more powerful inhibitors of FGF signalling including dominant-negative receptors of FGF4, chemical inhibitors of the FGF Receptor Tyrosine/Kinases, and FGF4 morpholino oligonucelotides have shown that FGF signalling is required for formation of neural tissue in *Xenopus* embryos (Hongo et al., 1999; Hardcastle et al., 2000; Delaune et al., 2005; Marchal et al., 2009).

Similarly, in the chick, inhibition of FGF signalling blocks neural induction by a grafted node (Streit et al., 2000) and in explanted tissue (Wilson et al., 2000). Strong evidence for a role in neural induction for FGFs have also come from a screen carried out to identify genes upregulated in response to 5 hours of signalling by a grafted node (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010). Of the ten genes found to be upregulated in response to 5 hours of signals from the node, seven can be induced by a source of FGF8, and their induction by a grafted node is abolished when FGF signalling is blocked but none of the 10 can be upregulated by any BMP antagonist tested (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010).

There is also evidence from experiments on both human and mouse embryonic stem cells, which have shown the requirement for FGF signalling to differentiate these cells towards a neuronal fate (Ying et al., 2003b; Kunath et al., 2007; Stavridis et al., 2007; Cohen et al., 2010; Sterneckert et al., 2010). While these studies suggest that an FGF signal is required for neural induction whether FGF alone can act as a direct neuralising factor has been more contentious (Stern, 2006). Several studies have suggested that FGF can act as a direct neuralising factor in chick (Rodríguez-Gallardo et al., 1997; Alvarez et al., 1998; Storey et al., 1998), *Xenopus* (Lamb and Harland, 1995; Hongo et al., 1999) and Zebrafish (Kudoh et al., 2004). Indeed, FGF has also been identified as the key endogenous neuralising factor in the ascidian *Ciona intestinalis*, a basal chordate, and therefore suggests an evolutionarily conserved role for the pathway in neural fate specification (Inazawa et al.,

1998; Darras and Nishida, 2001; Hudson and Lemaire, 2001; Kim and Nishida, 2001; Bertrand et al., 2003; Hudson et al., 2003). FGF signalling seems to be required for vertebrate neural induction (Sasai et al., 1995; Launay et al., 1996; Wilson et al., 2000; Linker and Stern, 2004; Delaune et al., 2005; Stavridis et al., 2007) but in *Xenopus* embryos FGF alone can only induce ectopic neural tissue in vitro (Linker and Stern, 2004; Delaune et al., 2005), and in the chick FGF is not sufficient to fully recapitulate endogenous neural fate specification – as the induced tissue is of a posterior character (Storey et al., 1998). FGF can only induce neural markers such as *Sox2* in explant culture assays (Wilson et al., 2000) but not in competent epiblast (Streit et al., 2000; Linker and Stern, 2004), although it is currently unclear why this difference exists. Thus, despite convincing evidence that FGF signals are required for neural induction, it seems that alone, FGF is not sufficient to account for the endogenous events of neural induction or the effect of node transplantation experiments, suggesting that additional signals are required.

Is a combination of FGF and BMP-inhibition sufficient for neural Induction?

FGF as BMP Inhibitor

In an attempt to reconcile the FGF data with the Default model, it has been suggested that both FGF signalling and antagonism of BMP together are required. Indeed several studies have suggested that one of the roles of FGF signalling in the context of neural induction is to function as a BMP inhibitor through inhibitory “cross-talk” between their intracellular signalling pathways (Pera et al., 2003). During canonical BMP signalling the BMP receptor Serine/Threonine protein kinases effect signalling by phosphorylating C-terminal residues of SMAD proteins 1, 5, 8 (Massague and Chen, 2000). This phosphorylation breaks an auto-inhibitory loop structure of the protein and enables the SMAD transcription factors to form complexes and translocate to the nucleus (Massague and Chen, 2000). However, FGF and Insulin Growth Factor signalling through receptor tyrosine kinases activate mitogen-activated protein kinase (MAPK) which can also interact with the SMAD1 protein and phosphorylate it at four conserved sites in the linker (middle) region of the

protein (Kretzschmar et al., 1997a; Pera et al., 2003) This phosphorylation event results in the degradation of the protein and thus the inhibition of the BMP signal as it recruits an E3 ubiquitin ligase that promotes polyubiquitination of the protein (Kretzschmar et al., 1997b; Pera et al., 2003). Though this signalling cross-talk was initially discovered in cultured cells (Kretzschmar et al., 1997b), Smad1-Linker mutant-constructs which are insensitive to MAPK mediated phosphorylation, are much more potent inhibitors of endogenous neural tissue in *Xenopus* microinjection studies and can also inhibit the ability of IGF2, FGF8 and Chordin to neuralise animal caps (Pera et al., 2003). Thus, it has been suggested that MAPK mediated FGF inhibition of BMP signalling enables cells to develop according to their default, neural fate, thereby explaining the requirement of FGF in other studies (Pera et al., 2003; Kuroda et al., 2005).

However, these studies do not provide evidence that the action of MAPK is solely through the SMAD1-phophorylation mechanism, and not by canonical MAPK signalling. Indeed, mouse knockouts with Smad1-MAPK phosphorylation resistant mutations are able to make it to term and have no obvious neural defects (Aubin et al., 2004) even though fibroblasts derived from these embryos remain resistant to FGF (MAPK) mediated repression (Aubin et al., 2004). This suggests that FGF signalling is required independently of MAPK-mediated BMP inhibition in neural induction. Indeed, mouse embryonic stem cells using a reporter for the expression of the neural marker *Sox1*, are able to differentiate along a neural lineage by MAPK mediated FGF signalling in the absence of increased Smad1-linker phosphorylation (Stavridis et al., 2007).

Interestingly, MAPK has also been implicated in the mechanism of neuralisation by dissociation of *Xenopus* animal cap cells (Kuroda et al., 2005). The dissociation followed by re-aggregation of *Xenopus* animal cap cells leading them to form neural tissue is one of the key experiments that lead to the formulation of the default model, as it was thought that the dissociation of the cells stopped BMP signalling (Muñoz-Sanjuán et al., 2002). In fact, subsequent evidence from RT-PCR and in situ hybridisation has

shown that the animal cap cells continue to signal through the BMP pathway even when dissociated (Kuroda et al., 2005). Furthermore, the act of dissociation actually causes the activation of the MAPK cascade that in turn causes phosphorylation of the Smad1-linker region (Kuroda et al., 2005). However, although this study does not address whether MAPK mediated inhibition of SMAD1 is the sole effect of neuralising the animal cap tissue, it does suggest that the idea of "default" differentiation is the result of previously unrealised signalling pathways that affects the choice between epidermal and neural cell fates. In summary, there is evidence for inhibitory cross-talk between FGF and the BMP pathway, but alone this is does not explain the requirement for FGF signalling in neural induction (Hongo et al., 1999; Hardcastle et al., 2000; Streit et al., 2000; Aubin et al., 2004; Delaune et al., 2005; Marchal et al., 2009). Such studies are important for they have begun to shed a light on how cells may integrate the effects of more than one signalling pathway, and perhaps provide a mechanism for cells to measure the relative levels of signalling pathways.

FGF & BMP-inhibitors.

Whether FGF signals in co-ordination with BMP-inhibitors are together sufficient for neural induction has also been contentious; The strongest evidence for the ability of FGF and BMP-inhibitors to act together as a combined signal for neural induction has come mainly from *Xenopus* microinjection studies targetting the A4-blastomere of cleavage stage embryos as an assay for neural induction. The advantage of this manipulation is that the A4 blastomere at the 32 –cell stage gives rise only to the ventral ectoderm far from the endogenous neural plate and can therefore be considered to be more isolated from the endogenous neuralising signals. In such studies injection of FGFs, or *Smad6* (an intracellular BMP antagonist) are unable to induce neural markers independently (Linker and Stern, 2004; Delaune et al., 2005). However, if a low concentration of FGF is injected in combination with a high concentration of *Smad6* into the A4 blastomere, neural but not mesodermal markers are ectopically expressed in the ventral ectoderm (Linker and Stern, 2004; Delaune et al., 2005), suggesting that a

combination of FGFs and BMP inhibition is sufficient for neuralisation of the ectoderm. Furthermore, these studies support studies in the chick (Streit et al., 1998) suggesting an early role of FGF followed by a later requirement for BMP inhibition, as FGF is required before gastrulation; treatment with SU5402 (a chemical inhibitor of FGF receptors) prior to gastrulation blocks neural induction in *Xenopus* embryos (Delaune et al., 2005). Additionally, the role of FGF cannot be explained solely by an inhibitory function of BMP signalling as SU5402 treatment cannot be rescued by co-injection BMP inhibitors (tBR, *Smad6* or Noggin) (Delaune et al., 2005).

A recent study in *Xenopus* embryos has also come to a similar conclusion for the sufficiency of BMP inhibition and FGF to induce ectopic neural markers, but in this case use of a single construct Smad5-somitabun (*Smad5-sbn*), thought to be a more potent inhibitor of BMPs, was sufficient to induce neural markers directly in ventral ectoderm (Marchal et al., 2009). Interestingly, although Smad5-sbn is thought to only inhibit BMP signalling (Hild et al., 1999), the ectopic expression of neural markers in ventral ectoderm by Smad5-sbn is blocked when FGF signalling is inhibited, either by a chemical inhibitor (SU5402) or by co-injection of dominant negative FGFR4 (Marchal et al., 2009). This suggests that active FGF signalling is required for the expression of neural markers in this assay, indeed, quantitative RT-PCR of whole embryo extracts suggest that FGF4 is upregulated after Smad5-sbn injection, although it is unclear whether the FGF is directly or indirectly induced. Nevertheless this study is consistent with other microinjection studies in *Xenopus* (Linker and Stern, 2004; Delaune et al., 2005) that suggests that FGF signalling in coordination with BMP inhibition is sufficient to induce ectopic neural markers in ventral ectoderm. Furthermore, like those studies the function of the pathways are separable, as two genes (*Zic3* and *Foxd5a*) normally upregulated in neural tissue are abolished by FGF inhibition but are not lost by either Noggin or Smad5-sbn microinjection (Marchal et al., 2009), thus adding further evidence to suggest that FGF signalling has BMP-independent roles in neural induction.

Despite the ability for BMP-inhibitors and FGFs together, to induce ectopic neural tissue in the ventral ectoderm of *Xenopus* embryos, even a combination of several FGFs (2,3,4,8) and BMP antagonists (Noggin, Chordin, *Smad6*, dnBMP receptor) together are not sufficient to induce the definitive neural marker, *Sox2* in the chick extra-embryonic region (Linker & Stern, 2004). How can we reconcile these findings? One possibility is that the injection of constructs at early cleavage stages of development may have unknown effects on cells, including affecting cell movements, or initiating a complex cascade of events that induce neural markers (Stern, 2006). For example, Smad5-sbn injections lead to the (direct or indirect) induction of FGF4 even though it is thought to act only in the BMP pathway (Hild et al., 1999; Marchal et al., 2009). Furthermore, FGFs are also potent inducers of mesoderm so its possible that this could be due to an indirect induction events, although use of FGF8b is in these studies is not without problem (Linker and Stern, 2004). FGF8 exists as several splice forms with different functions: FGF8b is a robust inducer of mesodermal fate in *Xenopus* (Hardcastle et al., 2000; Fletcher et al., 2006), whilst the FGF8a spliceform has been identified as a potent inducer of neural tissue (Fletcher et al., 2006). Thus, FGF8a may be a more appropriate candidate molecule for neural induction assays.

In summary, there is evidence for the requirement of FGF and BMP-inhibitors in neural induction, whilst there is evidence for an inhibitory interaction between FGF and BMP signalling (Kretzschmar et al., 1997a; Pera et al., 2003; Kuroda et al., 2005), it cannot explain all the roles of FGF (Linker and Stern, 2004; Delaune et al., 2005; Marchal et al., 2009). These experiments have begun to separate the timing at which signals are required, with an early role for FGFs and a later role for BMP inhibition emerging. However, given that no combination of FGFs and BMP inhibitors are sufficient to induce neural markers in the chick (Linker and Stern, 2004), other signals must be required.

Wnts and Neural Induction.

Adding further complexity Wnt has also been proposed as a neural inducing signal, although there are seemingly contradictory accounts of its involvement. Baker *et al.*, (1999) have suggested that Wnt signalling is able to induce a neural fate by antagonising *Bmp4* expression (Baker et al., 1999). In contrast, several studies have suggested that Wnts need to be inhibited for neural fate acquisition (Wilson et al., 2001; Aubert et al., 2002; Heeg-Truesdell and Labonne, 2006); in *Xenopus,* overexpressing Wnt inhibits the formation of the neural plate and in the chick cultured explants of early epiblast, neural fate is inhibited by Wnt signalling (Wilson et al., 2001). Furthermore, there is evidence in stem cells that Wnt inhibition can act a neuraliser, although only under specific conditions (Aubert et al., 2002; Verani et al., 2007), and several studies have suggested that one the first steps in response towards neuralisation is the expression of the Wnt inhibitor *Sfrp1* in cells (Aubert et al., 2002; Engberg et al., 2010).

How can these studies be resolved? Whilst it has been suggested that the difference in these studies could be due to the constructs used, or the concentration of Wnts involved (Heeg-Truesdell and Labonne, 2006), another interesting hypothesis is that we need to take into account the importance of timing (Stern, 2006). Indeed, Wnts are key dorsal determinants in the embryo and are important for specifying the dorsal tissues (including Spemann's Organizer). Later in development Wnts are involved in patterning tissue and are able to posteriorise tissue. Thus Wnt has several roles related to the formation of neural tissue during development; early in development β-catenin signalling is required to specify the dorsal side of the embryo, but later in development it needs to be inhibited for anterior neural tissue to develop.

Indeed, it has been suggested that canonical Wnt *(β-catenin*) signals are expressed in an organizer-like region of the *Xenopus* embryo termed the Blastula, Chordin and Noggin Expressing (BCNE) region, which is located in dorsal animal cells prior to gastrulation and formation of the organizer, and

contains both neurectoderm and Spemann organizer precursors (Kuroda et al., 2004). In this context β-catenin signalling is required for the expression of the anti-BMP proteins Chordin and Noggin which are required to predispose the prospective neurectoderm to induction by Spemann's Organizer (Kuroda et al., 2004).

The Wnt pathway has also been shown to interact with BMP signalling through a signalling cross-talk mechanism. In the absence of Wnt ligands binding to Frizzled receptors, the intracellular kinase: Glycogen Synthase Kinase 3 (GSK3) phorphorylates β-catenin thereby targeting it for degradation and prevents it binding to its co-factors (Tcf3) and entering the nucleus. However, GSK3 has also been shown to be able to hyper-phosphorylate the linker-region of SMAD1, adding two additional sites of phosphorylation to those mediated by (FGF/IGF-dependent) MAPK (Sapkota et al., 2007). This further enhances the rate of degradation of the SMAD1 signal (Sapkota et al., 2007). However, when Wnt signalling is active Dishevelled blocks GSK3 activity enabling β-catenin translocation and transduction of the Wnt signal, and also relieves the SMAD1 inhibition enabling BMP signalling to continue. Thus, it has been suggested that Wnt needs to be inhibited for neural induction as in the absence of Wnt that GSK3 is actively targeting SMAD1 for degradation and BMP signalling is blocked (Sapkota et al., 2007).

Even so, a combination of FGFs (2, 3, 4, or 8), together with BMP (*Smad6*, Chordin or Noggin) and Wnt antagonists (Cerberus, Dkk1, NFz8, Crescent) are still unable to induce the expression of the neural marker *Sox2*, directly in competent chick epiblast (Linker and Stern, 2004). This strongly suggests signals in addition to FGFs, BMP and Wnt inhibition must be required for neural induction.

Calcium

Another proposed neural inducing signal is an intracellular rise in calcium (Ca^{2+}) mediated by L-type Ca^{2+} channels. Experiments mainly in *Xenopus*

using chemicals that inhibit the action of Ca^{2+} channels or trigger an intracellular rise in Ca^{2+} have shown that inhibiting Ca^{2+} channels blocks neuralisation of dissociated animal caps (Leclerc et al., 2001), and that Noggin mediated neuralisation of animal caps induces an increase in Ca^{2+} (Moreau et al., 1994; Moreau and Leclerc, 2004b) which is necessary for its effect (Leclerc et al., 1997). Furthermore, a chemically induced flux of Ca^{2+} into animal caps cells alone is sufficient neuralise animal caps (Moreau et al., 1994), and visualisation of Ca^{2+} levels in vivo have shown that Ca^{2+} fluxes take place during gastrulation and are restricted to the *Xenopus* dorsal ectoderm (Leclerc et al., 2000; Moreau and Leclerc, 2004b).

However, although the mechanism of Ca^{2+}-induced neuralisation is unclear (it has been speculated that a rise in Ca^{2+} could activate the ubiquitously expressed Ca^{2+} dependent phosphatase enzyme *Calcineurin* (Saneyoshi et al., 2000) that might act to dephosphorylate SMAD1 and inhibit BMP signalling (Moreau and Leclerc, 2004a; Moreau and Leclerc, 2004b), although there is no direct evidence for this), a recent study has uncovered a key player in modulating Ca^{2+} levels during neural induction, *Calfacilitin* (Papanayotou, unpublished observations). *Calfacilitin* is one of the genes identified in a differential screen for genes up-regulated in response to 5 hours of exposure from a grafted node and encodes a novel transmembrane Ca^{2+}channel facilitator that is able to potentiate Ca^{2+} fluxes by L-type Ca^{2+} channels (Papanayotou, unpublished observations). Furthermore, morpholino-mediated knockdown of *Calfacilitin* blocks neural induction by a grafted node, but not, early response genes (*Sox3* and *ERNI*). This study therefore supports findings in *Xenopus* for the requirement of Ca^{2+} flux in neural induction, and also for the first time identifies a gene induced by the node that can effect Ca^{2+} fluxes. Furthermore, *Calfacilitin* is upregulated by FGF, a signal that has also been reported to activate L-type Ca^{2+} channels in *Xenopus* (Lee *et al*., 2009). Thus, although the precise mechanism of Ca^{2+} fluxes is unknown, it is clear that a change in the Ca^{2+} levels in the prospective neural tissue is required and that part of the neural induction cascade, initiated by signals from the node, induces proteins that potentiate the action of Ca^{2+} channels at the cell membrane. However, given that FGF

is able to induce *Calfacilitin* and Ca^{2+} fluxes, Ca^{2+} might be an unlikely candidate to be the missing signal in the neural induction assays.

Protein Kinase C

The balance of protein kinase C (PKC) to cAMP has also been implicated in stimulating neural specification in amphibians (Otte *et al*., 1988; Otte *et al*., 1989; Otte *et al*., 1992; Otte and Moon, 1992), although how this might be involved in known signalling pathways is unclear. One recent study has identified a secreted factor in *Xenopus* - Syndecan 4 (Syn4), this protein can signal through a PKC-dependent pathway and has neuralising activity (Kuriyama and Mayor, 2009). Syn4 is a heparin sulphate proteoglycan able to interact and bind growth factors, it is known to interact with both the FGF (MAPK) pathway, as well as through the Syn4-PKC-dependent pathway (Otte and Moon, 1992; Horowitz and Simons, 1998; Horowitz *et al*., 1999; Bass *et al*., 2007; Matthews *et al*., 2008)

Syn4 is expressed in the dorsal ectoderm and its expression becomes restricted to the neural plate (Kuriyama and Mayor, 2009), and morpholino's against Syn4 inhibit endogenous neural plate formation. Interestingly, overexpression of Sny4 can induce neural markers (*Sox2* and *Sox3*) in the ventral ectoderm when injected into the A4 blastomere at the 32-cell stage (Kuriyama and Mayor, 2009). Because this induction is lost when dnFGF is co-injected, or the embryos are treated with SU5402, some of the activity of Sny4 must be through modulation of the FGF pathway, however injection of PKCα can also induce neural tissue in this assay in an FGF-independent manner although its mechanism is unclear (Kuriyama and Mayor, 2009). Thus, PKC signalling mediated by Syndecan4 has also been implicated as a neural inducing signal, though whether Syn4 acts through the FGF-dependent or -independent pathways during development is unknown and remains an intriguing molecule in the context of neural fate decisions.

Retinoic Acid

Retinoic acid (RA) is the active retinoid in developing embryos. It is active because it can enter the nucleus and activate or represse genes by binding to ligand-activated transcription factors. Retinoic acid is synthesized from vitamin A (retinol) found in yolk, or the maternal circulation in mammals. To date little is known about any role in neural induction, despite its well described roles in other developmental contexts including patterning. However, it is known that several retinoic acid synthesizing enzymes are expressed in and around the node including: *Raldh3* in the node, and *Raldh2* in the mesoderm (Blentic *et al*., 2003). Recent studies have identified an important role for retinoic acid in controlling the onset of neural differentiation in embryonic stem cells (Engberg *et al*., 2010; Stavridis *et al*., 2010) and axis elongation in the chick (Stavridis *et al*., 2010)

It is known that mouse ES cells will differentiate spontaneously towards neurectodermal fate in serum-free, adherent monocultures, and because of the minimal medium conditions in which cells were cultured it had been suggested that cells differentiate by default (Tropepe *et al*., 2001; Watanabe *et al*., 2005; Smukler *et al*., 2006; Lenka and Ramasamy, 2007; Kim *et al*., 2009). However, Enberg *et al*., (2010) now show that retinoic acid is actively synthesized by ES cells from vitamin A in the medium, if vitamin A is then removed from the medium, or RA synthesizing enzymes in the cultures are blocked, the differentiation event is inhibited (Engberg *et al*., 2010). Furthermore, RA signalling in stem cells has also been linked to regulation of FGF signalling as RA treatment of mES cells has been shown to cause a short but rapid induction of FGF followed by a decrease in FGF over the first 5 days of differentiation (Stavridis *et al*., 2010). Interestingly, both FGF and RA signals are required for the expression of *Sox1*, a late neural marker, in cultured cells suggesting that the RA regulation of FGF (induction followed by inhibition) may be important (Stavridis *et al*., 2010).

An analogous signalling mechanism involving RA and FGF has also been suggested to play a role regulating in neural differentiation in axis elongation

in the chick (Stavridis *et al*., 2010). During neurulation in the chick the spinal chord elongates due to growth at the tail end of the embryo known as the stem zone. Here too, cells must transit from a self-renewing population to differentiate when they leave the epiblast population. Similarly, in this context RA attenuation of FGF signalling also promotes differentiation as cells leave the stem zone, by regulating *Sox1* expression (Stavridis *et al*., 2010).

Thus, recent studies have identified a role for RA in regulating the transition of cells from a state of pluripotency towards neural fate acquisition by the context dependent regulation of FGF. In light of the fact that RA synthesizing enzymes are expressed in the node, RA could be considered as an excellent candidate for a missing signal in neural induction.

Notch Signalling

Notch is unique in the signalling pathways considered so far as it is mediated by cell-cell contact rather than involving a diffusible, secreted ligand. Notch has well characterised roles in specifying neuronal fates in the fly, but until very recently little was known about potential roles for it in vertebrate neural induction. Two context-dependent cellular behaviours have been attributed to Notch signalling termed lateral inhibition and lateral induction. These processes effect the formation of boundaries and the coordination of cell fate decisions in populations of cells, respectively, both of which have been implicated in three recent studies with implications for neural induction.

During gastrulation the primary germ layers that constitute the three major cell lineages are formed by the ingression of the future mesoderm and endoderm cells from the surface ectoderm. Revinski *et al*., (2010) using injection constructs to intra-cellularly overexpress ($Notch^{ICD}$) or inhibit ($su(H)1^{DBM}$) Notch signalling, have shown that Notch signalling regulates the boundary between the segregating germ layers in *Xenopus* (Revinski et al., 2010). Overexpression of Notch expands the neural territory of *Sox2*-expressing cells at the expense of the *Brachyury*-expressing mesoderm, whilst the loss of

Notch causes the opposite effect (Revinski *et al*., 2010). Importantly not all injected cells are affected by the manipulation and many retain their correct positional fate, suggesting that it is boundary formation, not specification that Notch is controlling in this context. This study highlights a conserved role for Notch in the separation of germ layers during development, although interestingly in zebrafish (Kikuchi *et al*., 2004) and sea urchins (Sherwood and McClay, 1999; Sherwood and McClay, 2001), the effects of Notch are reversed, and Notch signalling enhancing mesoderm. Nevertheless, regulation of Notch signalling is clearly an important step in neural fate decisions.

Interestingly, a role in the coordination of early neural cell fate decisions of Notch has also been suggested from a study in embryonic stem cells (Lowell *et al*., 2006). Standard differentiation protocols using growth factors including FGF, generates a low proportion of cells that will differentiate into neurones, although conditions to optimise protocols have been developed they still result in a heterogenous cell population, with some cells remaining in a pluripotent undifferentiated state. However, by activating Notch in human and mouse ES cells, a substantial increase in the proportion of cells that differentiate occurs (Lowell et al., 2006). Furthermore, genetic or chemical inhibition of Notch signalling prevents factors that would normally induce neural differentiation from doing so (Lowell *et al*., 2006). Thus, these findings strongly implicate Notch signalling as a key component in coordinating the transition from pluripotent self-renewing state to a state of neural differentiation.

Notch has also been implicated in controlling the transition of self-renewal to neural differentiation in the tail-bud “stem zone” during axis elongation in the chick (Akai *et al*., 2005). The tail-bud proliferative region drives the growth and elongation of the embryo, as cells exit this region they differentiate to both neural and non-neural fate. In this context, FGF and Notch signalling are required to maintain the self-renewal state, but a Notch dependent lateral-inhibition-like event has been proposed to account for how cells acquire neural fate upon leaving the region as retinoic acid decreases the level of

FGF, and cells up-regulate *Delta-1* expression and down-regulate Notch, resulting in the acquisition of neuronal fate (Akai *et al*., 2005).

Together these studies suggest important roles for Notch in neural fate decisions, a Notch signalling is required early in a lateral induction-like role to coordinate neural differentiation, but at later stages lateral-inhibition might be involved in both the formation of germ layer boundaries, but later needs to be downregulated for neuronal differentiation to continue. Furthermore, it reinforces the observation that a single signalling pathway can have multiple, even opposing roles, during development.

Insulin Growth Factors

The final signalling pathway implicated in neural induction is the Insulin Growth Factor (IGF) pathway. IGF can signal through a similar pathway to FGF, via MAPK, thus they are also able to inhibit BMP signalling through MAPK-mediated SMAD1 phosphorylation events (Pera *et al*., 2003). Injection studies in *Xenopus* have shown that IGF is a potent inducer of anterior neural fate, as overexpression of IGF2 in 1-cell at the 2-cell stage leads to the ectopic formation of anterior structures including ectopic eyes (Pera *et al*., 2001) which are lost if IGF signalling is blocked by injection of a dominant negative IGF receptor (Pera *et al*., 2001). Finally, animal caps cut from embryos injected with IGF2 or IGFBP-5 (IGF-Binding Protein 5) express anterior neural markers (*Pax6, Six3, Rx2a, Otx2*), but not more posterior markers, such as the midbrain marker *En2* (*Engrailed 2*) (Pera *et al*., 2001). Given these findings, it has been suggested that the main activity of IGF in these studies is through BMP inhibition, however, the BMP-independent role is less clear.

It has been reported (Wilson *et al*., 2000) that chick explants from the early pre-streak epiblast (prospective neural) a time prior for the formation of the node are able express neural markers when cultured in vitro. However, in contrast, it has been found that epiblast tissue cannot express neural markers

in isolation from signals from the node until much later in development (Garcia-Martinez *et al*., 1997), nor are the known signals expressed at this time in the epiblast sufficient to induce definitive neural plate markers in the area opaca epiblast (Streit *et al*., 2000; Albazerchi and Stern, 2007). One difference between the in vitro studies is the method of culture, the latter cutting transverse sections of gastrula stage embryos, the former small explants requiring N2 supplement which contains a defined medium of factors including IGF. Given that IGF signalling has been identified as a neuralising factor, it is possible that IGF signalling in the medium is responsible for the neuralisation of tissue.

In summary, since Spemann and Mangold identified the Organizer researchers have tried to identify the instructive signals that direct neural fate acquisition. Despite, much of emphasis focused on the concept of a "default" neural fate in both *Xenopus* and stem cells that occurs the absence of BMP signalling, there is now convincing evidence that BMP-inhibition alone is not sufficient for neural induction and that other key signalling pathways are involved including FGFs and Wnts. However, some combinations of signals are able to induce neural markers under certain experimental conditions in *Xenopus*, including the animal cap ectoderm, though no combination of signalling molecules has yet been able to induce the definitive neural marker, *Sox2,* in competent chick epiblast, strongly suggesting that further signals than these are required. Furthermore, the complexity of this system is enhanced by interactions between pathways and the different roles a single signal can have. An important way of understanding neural induction is to consider the timing and responses of neural tissue, which may provide us with valuable insight into the events of neural induction as a developmental process.

The Timing of Neural Induction

To understand neural induction it is crucial to identify when the process of neural fate acquisition occurs so that misexpression studies and loss of

function studies can be properly interpreted. In the chick, node transplantation experiments have revealed a time-window in which the node can induce neural fates; from stage 3^+ until stage 4 (Hamburger and Hamilton, 1951) it losses its ability to induce a fully patterned nervous system (Storey *et al*., 1992). Competence to respond to a node in host tissue is lost between stages 4 and 4^+, a finding that suggests that the induction of a fully patterned CNS induced by the organizer normally ends by these stages (Gallera and Ivanov, 1964; Gallera and Nicolet, 1969; Gallera, 1970; Gallera, 1971a; Dias and Schoenwolf, 1990; Storey *et al*., 1992; Garcia-Martinez et al., 1997; Darnell et al., 1999). Similarly, in amphibians, competence of the ectoderm to respond to neural induction is thought to be lost at the end of the gastrula stage, between stages 12 and 13 (Waddington and Needham, 1936; Gurdon, 1987; Sharpe and Gurdon, 1990; Servetnick and Grainger, 1991).

However, due in part to the classical Spemann organizer transplantation experiments (Spemann and Mangold, 1924), research in neural induction has been considered as a process exclusively enacted through signalling by the organizer. Therefore by this logic, the formation of the organizer during gastrulation should mark the time at which neural induction begins. However, studies in the chick have suggested that the earliest step in neural induction occurs prior to gastrulation, and is marked by the expression of *ERNI* and *Sox3*, a step for which FGF signalling is sufficient and required (Streit et al., 2000; Wilson et al., 2000). Similar conclusions for an early event involving FGF, have also been reached for amphibians (Kuroda et al., 2004; Delaune et al., 2005).

The Source of Neural Induction Signals.

Given that neural induction begins prior to the formation of a morphological organizer, what is the source of early neural induction signals? One possibility is that the cells that will later form the organizer provide the signals. Prior to gastrulation the cells that give rise to the organizer exist in two cells populations; “posterior cells” located at a crescent-shaped ridge termed Koller’s sickle that lies at the posterior edge of the epiblast, and “central cells”

which are present in the epiblast (Izpisúa-Belmonte et al., 1993; Hatada and Stern, 1994). However at this stage only posterior cells have low neural-inducing ability, whilst central cells have none (Izpisúa-Belmonte et al., 1993; Tam and Steiner, 1999; Streit et al., 2000), as assessed by grafts into the area opaca of a host embryo. However, the posterior cells in Koller's sickle, are unlikely to be responsible for induction of early neural markers in the epiblast (Streit et al., 2000), and it is not until later in development when these cell populations meet and unify to form the morphological node, that they have full neural inducing ability (Waddington, 1930; Izpisúa-Belmonte et al., 1993). Similarly in *Xenopus*, the blastula Chordin- and Noggin-expressing cells (BCNE) have been reported (Kuroda et al., 2004) to have similar properties to the "posterior" group of cells, although in the chick Noggin is not expressed until after the neural plate formation has begun (Streit et al., 1998).

If organizer precursors do not provide the signal that initiates neural induction events what else could be the source? Prior to gastrulation, two sources of FGFs have been reported, *FGF3* in the cells of the epiblast itself (although this has only been confirmed by RT-PCR, and is not at a level observable by in situ hybridization) (Wilson et al., 2000), and a more likely candidate, *FGF8* expressed in the hypoblast tissue (Albazerchi and Stern, 2007)- a layer that comes to underlie the area pellucida epiblast. The hypoblast being the source of these early signals is supported by the fact that hypoblast tissue, grafted to the competent area opaca epiblast, can induce the ectopic expression of markers seen in the epiblast including *Sox3* and *ERNI*, but can do so only transiently (Foley et al., 2000; Streit et al., 2000; Knezevic and Mackem, 2001). Together these results suggest that the earliest source of signals for neural induction originate in a combination of the hypoblast (anterior visceral endoderm in the mouse) (Thomas and Beddington, 1996; Belo et al., 1997; Varlet et al., 1997; Beddington and Robertson, 1999) and organizer precursor cells. However, neither of these cell populations hypoblast or "posterior" cells, are able to induce the definitive neural marker *Sox2*. Thus the signals that enable prospective neural cells in the epiblast expressing the early neural markers such as *Sox3* and *ERNI* to continue to acquire full neural plate character remains unknown.

Is the node required for neural fate?

The fact that tissues other than the organizer have inductive properties, raises the possibility that more than one organizing region exists. Two models for the formation of a fully patterned nervous system, have been suggested, the 'activation/transformation' model of Nieukwoop (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) proposes that the nervous system is initially induced with 'anterior' (forebrain) character, and that later signals 'transform' parts of it to more caudal fates. However, Mangold (Mangold, 1933) proposed that multiple organizers may exist for the head, trunk and tail regions of the axis. The two most compelling cases for additional organizers come from work in mouse and in zebrafish.

In the mouse it has been proposed (Beddington, 1994) that the anterior visceral endoderm (AVE), (an extra-embryonic layer equivalent to that of the hypoblast) can act as the proposed "head organizer" (Mangold, 1933). Experimental evidence for this role comes from the observations that the AVE is required for head formation (Thomas and Beddington, 1996; Varlet et al., 1997; Acampora et al., 1998; Dufort et al., 1998), and that HNF3β knockout mice lacking a node (Ang and Rossant, 1994; Weinstein et al., 1994; Dufort et al., 1998) still retain neural tissue. However, despite being required for head development the AVE does not possess neural-inducing activity in grafting experiments unless combined with prospective organizer tissue, and is only able to induce cells in competent regions (prospective forebrain regions) (Tam and Steiner, 1999; Robb and Tam, 2004). These studies suggest a permissive, or indirect role in neural induction for the AVE, which is consistent with data from hypoblast experiments in the chick that suggest a transient induction of early neural markers (Foley et al., 2000; Albazerchi and Stern, 2007).

In the zebrafish, it has been suggested that the shield and the more ventral marginal region emit signals responsible for inducing the nervous system in the head and in the trunk/tail region, respectively (Agathon et al., 2003; Furthauer et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004). Indeed it has been suggested that a gradient of organizer ability exists throughout the

entire blastula-gastrula margin, and that grafts from any region can generate ectopic axial structures when transplanted into the animal pole of a host embryo (Fauny et al., 2009). However, it is only the dorsal region, the embryonic shield, that is able to induce a fully patterned secondary axis in including anterior structures (Fauny et al., 2009). Thus, this result may reflect the gradient of signals that patterns the cells at the margin, so that those closest to the shield are exposed to, and express similar signals to the "true" organizer region. However, whilst there is an argument for just one organizer having full neural induction ability, it is the case that there are regions of the nervous system that are never close to the organizer like the trunk/tail region of the zebrafish and the most anterior neural tissue in chick. Thus combinations of signals emanating from non-organizer tissues at different times during developmental times might account for these tissues acquiring a neural fate.

In summary, to date only the gastrula-stage node in amniotes, the dorsal lip in amphibians and the shield in teleosts have been shown to be "true" organizers, capable of inducing a complete ectopic nervous system. However, tissues other than the organizer do release signals that complement the function of the organizer, either through directly inducing genes, or providing signals that pattern the induced tissue.

Cell fate choices of Neural Tissue.

Due to the classical Spemann transplantation experiments (Spemann and Mangold, 1924), neural induction has often been considered as a choice between epidermal and neural fates, as in this experiment signals from the transplanted organizer convert prospective (ventral) epidermis to a neural fate. However, this is an experimental paradigm that reflects the ability of the organizer to convert ectodermal tissue to a neural fate, and not necessarily the events that occur in prospective neural cells acquiring neural fate. One study (Sheng et al., 2003) has shed light on the cell fate decisions that take place in prospective neural cells prior to the formation of the neural plate in the context of gastrulation. Prior to gastrulation the precursors of all three germs layers exist in a pluripotent epiblast layer, from which prospective

mesendodermal cells will ingress through the primitive streak. Once ingression ceases, the cells that have remained in the epiblast will become neurectodermal cells. Sheng *et al.,* (2003) isolated a zinc-finger transcriptional activator termed *Churchill* from a screen for early responses to neural induction. *Churchill* is expressed in prospective neural tissue towards the end of gastrulation, and in turn regulates the expression of SIP1, a transcriptional repressor (Sheng et al., 2003). Through *Sip1*, *Churchill* down-regulates the expression of *Brachyury,* which is essential for cell ingression through the primitive streak, thus retaining neural cells in the epiblast - a role that has been confirmed in both chick (Sheng et al., 2003) and zebrafish (Londin et al., 2007). Thus, one of the early steps in neural fate acquisition is a choice between neural and mesendodermal fates, and it is *Churchill* through SIP1 that is instrumental in defining the boundary between these fates.

The fact that one of the early steps in prospective cells is to express transcriptional repressors of mesodermal genes suggests that neural induction needs to be considered in the context of the events during gastrulation. Ectodermal and mesodermal fates, which both share a boundary with neural tissue are induced in response to TGFβ signals, with BMP acting through SMAD1 to induce ectodermal fates and Nodal/Actvin acting through SMAD2 to induce mesodermal fates. A study by Chang and Harland (2007), have shown that the inhibition of *Smad1* and *Smad2* can induce ectopic neural markers in the ventral ectoderm of *Xenopus* embryos and that stimulation of *Smad2* in the neural plate inhibits its formation by converting neural tissue to neural crest and mesodermal fates (Chang and Harland, 2007). Thus, neural fate decisions during gastrulation can be seen as a choice between neural and ectodermal fates, mediated in part by the regulation of TGFβ signalling, therefore for efficient neural induction, suppression of both Smad1 and Smad2 (ectodermal and mesdodermal fates) are required. However, whilst this study shows the sufficiency for inhibition of *Smad1* and *Smad2* to induce ectopic markers in the ventral ectoderm, it does not show whether FGF signalling is required for this effect, as has been reported (Linker and Stern, 2004; Delaune et al., 2005; Marchal et al., 2009) for all previous studies involving inhibition of BMP signalling.

The Transcriptional Response to Neural Induction.

Many studies have focused on understanding neural induction with the goal of identifying the instructive signals that specify neural tissue. However, understanding the early genetic cascade in cells undergoing neural fate acquisition prior to the formation of the neural plate can also provide us with an understanding of the timing and mechanisms involved. Much progress in our understanding of the early transcriptional responses to the signals of neural induction has come from work in the chick (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010).

Time-course studies have revealed the requirement of at least 12 hours of exposure to signalling from a grafted node for a competent region of the chick epiblast, the area opaca, to form an ectopic neural plate (Gallera and Ivanov, 1964; Gallera, 1971b). This time coincides with the expression of *Sox2* a basic Helix-loop-Helix transcription factor expressed throughout the neural plate and conserved across vertebrate species. Thus, in the context of the transcriptional cascade, *Sox2* is a crucial gene, as it marks the time at which cells have received sufficient signals to commit to a neural fate, and therefore has been considered a "definitive" neural plate marker (Stern, 2005). However, as no combination of factors tested, including combinations of FGFs, BMP and Wnt inhibitors, have been able to induce *Sox2* expression in the chick (Streit et al., 2000; Sheng et al., 2003; Gibson et al., 2010), research has focused on identifying the genes that are expressed upstream of *Sox2* as a method of identifying the missing signals.

A differential screen for genes up-regulated in response to the first 5 hours of signals identified 7 genes, all of which have been characterized and shown to be expressed in prospective neural tissue and induced by FGF (Streit et al., 2000; Sheng et al., 2003; Gibson et al., 2010). The earliest genes to be expressed in response to node transplantation experiments *Sox3* and *ERNI* (Streit et al., 2000) are markers of prospective neural cells or a "pre-neural" state, which are not yet fully committed to a neural fate. The expression of *Sox3* in the prospective neural tissue is also conserved across vertebrates (Rex et al., 1994; Uwanogho et al., 1995; Rex et al., 1997; Wood and

Episkopou, 1999).

Several of the remaining genes expressed in response to 5 hours of signals from a grafted node, including *churchill*, *Dad1, polyubiquitin, and ferritin heavy chain* seem to have a protective functions for neural tissue, *Churchill* (Sheng et al., 2003) as previously described, protects prospective neural cells from mesodermal fates, whilst *Dad1, polyubiquitin, and ferritin heavy chain* (Gibson et al., 2010) are all genes involved in controlling programmed cell death. However, irrespective of their function, the fact that they are induced by FGF does not provide the signal that is sufficient to induce the onset of *Sox2* expression (Streit et al., 2000; Linker and Stern, 2004).

The complexity of regulatory events involved in the expression of *Sox2* has been shown by analysis of its cis-regulatory region (Uchikawa et al., 2003). Although *Sox2* is a pan neural marker it is regulated by 5 different enhancer regions, with each enhancer containing numerous binding sites and responsible for a different domain of its expression (Uchikawa et al., 2003). The N2 enhancer is responsible for the onset of expression of *Sox2* in the neural plate (Uchikawa et al., 2003), recently a mechanism for its induction has been reported (Papanayotou et al., 2008) involving two genes, BERT and ERNI (Streit et al., 2000; Papanayotou et al., 2008). ERNI, as previously described, is one of the first genes expressed in response to signals from a grafted node, and is expressed in the pre-streak epiblast. However, Papanayotou et al., (2008) have shown that one of its functions is to form an inhibitory complex with the chromatin remodelling proteins Brahma and HP1**γ** at the N2 enhancer, thereby blocking the transcription of *Sox2* (Papanayotou et al., 2008). However, BERT expressed in tissue surrounding to the node at stage $4/4^{+}$ is able to break this inhibitory complex by binding to ERNI, thereby enabling the expression of *Sox2* in the neural plate (Papanayotou et al., 2008). Thus, somewhat paradoxically, one of the first steps in prospective neural cells is to inhibit expression of the definitive neural marker *Sox2*, although this is also primes the genes for a later onset of expression triggered by BERT. However, BERT, which requires 11-12 hours of exposure to neural inducing signals, is not induced by FGF, BMP or Wnt inhibitors (Papanayotou

et al., 2008), therefore, identifying what regulates it, would identify one of the key signals in the onset of neural plate commitment.

In conclusion, our developing understanding of neural induction is of a complex cascade of interacting events and signalling pathways, by which prospective neural tissue is instructed to a neural fate. Whilst several key signalling pathways have been identified, including FGF and BMP inhibition, there is difficulty in reconciling the relative importance and sufficiency of these pathways due to differing results in chick explants studies, *Xenopus* animal caps assays and in vitro assays. However, no combination of signals has yet been identified that are sufficient to induce an ectopic neural plate in the chick, which strongly suggests that additional signal must be involved. Furthermore, there is still much we don't understand including the mechanism of response of neural cells to BMP inhibitors, the cell state of cells in prospective neural cells as they are induced towards a neural fate, and the cell fate decisions made in early neural induction events.

Thesis Aims

Given these conclusions this study aims to investigate the early steps of neural induction by addressing three questions:

1) What are genes are up-regulated in prospective neural cells during the early stages of response to neural induction, and what signals control their expression?
2) What are the mechanisms by which prospective neural cells can respond to changing levels of the TGFβ pathway during early development?
3) What is the ground-state of specification of the pre-streak epiblast in cells at the start of the neural induction cascade?

Chapter Two

Methods & Materials

In Vitro Culture of Chick Epiblast Isolates

Fertilized hens' eggs (Brown Bovan Gold, Henry Stewart, UK) were obtained for collagen embedded culture of dissected epiblast tissue, as previously described (Streit et al., 1998). Eggs were incubated at 38°C to the stage required. Pre-primitive streak stage chick embryos were staged according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976) and obtained by cutting the vitelline membrane surrounding the embryo before transferring into Tyrode's saline solution (80g NaCl, 2g KCl, 2.71g $CaCl_2.2H_2O$, 0.5g NaH_2PO_4, 2g $MgCl_2.6H_2O$, 10g glucose, H_2O to 1 L). Embryos were freed from the vitelline membrane with forceps and the yolk cleaned away with streams of Tyrode's solution to reveal the area opaca. Embryos were placed dorsal side up and the hypoblast layer of cells carefully removed using tungsten needles. Pieces of epiblast were cut from the middle and anterior-lateral regions of the epiblast with tungsten needles and transferred, individually, to a 35-mm plastic dish in 4 µl drops of Tyrode's solution. A 10 µl drop of collagen solution (700 µl Collagen-I (Invitrogen), 100 µl Medium 199, 100 µl 1M $NaHCO_3$, 10 µl Penicillin-Streptomycin (100x) (Invitrogen), 10 µl N2 supplement (Invitrogen), 63 µl H_2O, 7 µl 1 M NaOH) was then added to each dissected epiblast and transferred to a new 35-mm Petri-dish. The dish was inverted to allow the explanted tissue to embed at the apex of the collagen drop, and incubated at 38°C for 30 minutes to harden. Once hardened, 3 ml of N2 supplemented medium (1 ml Medium 199, 1 ml $NaHCO_3$, 100 µl Glutamax, 100 µl Penicillin-Streptomycin, 100 µl N2 supplement, H_2O to 10 ml - additional chemical inhibitors as appropriate to the experiment) were added to the culture dish which was placed in a 37°C 5% CO_2 incubator for either 40 hours or 6 days. For day 6 cultured explants, the medium was changed after 3 days. For positive and negative controls for the in situ hybridization, embryos from a range of stages were also isolated and

pieces of stage 4 primitive streak (primitive streak and later stage embryos were staged according to (Hamburger and Hamilton, 1951)) dissected and processed for *in situ* hybridization.

Anti-sense RNA probes for Chick whole mount in situ hybridization

The plasmids from which labelled RNA probes were synthesized for in situ hybridization are listed in Table 2.1. Probes were synthesized from approximately 3 µg of plasmid DNA and linearized using the appropriate restriction enzyme. The antisense sequence was then transcribed with either T7, T3 or Sp6 polymerase (Promega) in the presence of digoxigenin-labeled dUTP (Roche) to make a labelled anti-sense transcript.

Probe	Length bp	Marker For	Reference	Kind Gift Of
Brachyruy	350	Primitive streak mesoderm, notochord	(Smith et al., 1991)	V. Cunliffe
Bert	750	Early neural plate	(Papanayotou et al., 2008)	
Dad1	500	Neural plate	(Gibson et al., 2010)	
δ-crystallin	750	Lens	(Alemany et al., 1989)	F. de Pablo
Dlx5	667	Pre-placode region	(Ferrari et al., 1995; Streit, 2002; McLarren et al., 2003)	G. Lizarraga
ERNI	950	Prospective neural, neural plate border	(Streit et al., 2000)	

Eya2	450	Pre-placode region	(Mishima and Tomarev, 1998)	S. Tomarev
Gata2	900	Non-neural ectoderm	(Sheng and Stern, 1999)	
Gata3	700	Neural plate border	(Sheng and Stern, 1999)	
L-Maf	800	Lens	(Ogino and Yasuda, 1998)	H. Ogino
Msx1	800	Neural plate border	(Liem et al., 1995; Streit and Stern, 1999)	K. Liem
Pax2	800	Otic Placode	(Baker and Bronner-Fraser, 2000)	D. Henrique
Pax6	688	Anterior neural plate, lens development	(Goulding et al., 1993; Bhattacharyya et al., 2004)	A. Bang
Pax7	660	Neural crest	(Kawakami et al., 1997)	A. Kawakami
Six4	750	Pre-placode marker	(Esteve and Bovolenta, 1999)	A. Streit
Slug	950	Neural crest (Nieto et al., 1994)	(Nieto et all., 1994)	D. Wilkinson
Sox1	600	Late Neural Marker	(Kamachi et al., 1998)	M. Uchikawa
Sox2	1700	Definitive neural plate marker	(Kamachi et al., 1998)	P. Scotting
Sox3	825	Prospective neural marker	(Uwanogho et al., 1995)	P. Scotting
TrkC	800	Early neural plate		

Table 2.1. Alphabetical list of chick RNA probes used for in situ hybridization.

Whole mount in situ hybridization on chick explants

The expression of various genes in cultured epiblast tissue explants was assessed by a modification of the whole-mount in situ hybridization method as previously described (Streit et al., 1995; Théry et al., 1995; Streit et al., 1997). Due to their small size, washes on explanted tissue were carried out in mesh baskets using an automated in situ hybridization machine.

In brief, explants were fixed in 4% paraformaldehyde containing 2 mM EGTA in PBS (Phosphate Buffered Saline: 8.76 g NaCl, 2.14 g $N_2HPO_4.7H_2O$, 2.3 g $Na_2H_2PO_4$ in 1 Litre H2O) (pH 7.0) overnight at 4°C. After fixation explants were washed in PBS once, the embedded tissue was dissected out from the collagen, then dehydrated in 100 % methanol to be stored at – 20°C overnight. Explants were progressively rehydrated in PTW (Phosphate Buffered Saline; 0.1 % Tween-20 solution) before washing in 1:1 PTW:hybridization solution, then finally placed into hybridization solution (50% Formamide, 1.3x SSC (pH 5.3), 5mM EGTA, 50 µg/ml yeast RNA, 0.2% Tween-20, 0.005% CHAPS, 100 µg/ml Heparin). Explants were loaded into small vials in a 500 µl volume of hybridisation solution (approximately 10-20 explants per column) and incubated for 3 hours in a 70°C water bath. The hybridization solution was then replaced with the appropriate antisense probe and allowed to hybridise overnight at 70°C. Post-hybridisation, three rinses were carried out with warmed hybridization solution, followed by two washes of 30 minutes each and a final heated wash of 20 minutes with a 1:1 hybridisation:TBST (Tris-Buffered Saline Tween-20: 1.4 M NaCl, 27mM KCl, 0.25 M Tris-HCl pH7.5, 1% Tween-20) solution. The mesh baskets containing the explanted tissue were then transferred to a BioLane™ HTI 16V automated in situ machine (Intavis) for all subsequent washes including: TBST rinses (three washes of 1 hour at room temperature), blocking (5% heat inactivated goat serum and 1 mg/ml bovine serum albumin in TBST), antibody incubation (Anti-digoxigenin alkaline phosphatase (Roche) overnight at 4°C) and post-antibody TBST washes (4 washes of 1 hour at room temperature). Explants

were removed from their baskets into 6-well plates and washed twice in NTMT (0.1M, NaCl, 0.1M Tris-HCl (pH 9.5), 0.005M $MgCl_2$, 0.1% Tween-20) Solution for 10 minutes. Finally, explants were incubated in NTMT with 4.5 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate: 7.5 mg/ml in 70% dimethylformamide) and 3.5 μl NBT (Nitro-blue teratzolium: 50 mg/ml in 100% dimethylformamide) per ml, in the dark, until the colour reaction had developed satisfactorily. To stop the reaction, embryos were washed in PTW and fixed in 4 % formaldehyde in PBS (pH 7).

Chick embryo New cultures

Fertilized hens' eggs were incubated at 38°C for 16-18 hours to obtain stage 3^+ / 4 embryos (Hamburger and Hamilton, 1951) for factor testing experiments and cultured, as previously described, in modified New culture (New, 1955; Stern and Ireland, 1981). The shells were opened and the yolks extracted and floated in Pannett-Compton (PC) saline solution (Pannett and Compton, 1924) (40 ml Solution A [121g NaCl, 15.5g KCl, 10.42g $CaCl_2.2H_2O$, 12.7g $MgCl_2.6H_2O$, H_2O to 1 L], 60 ml Solution B [2.365g $N_2HPO_4.2H_2O$, 0.188g $NaH_2PO_4.2H_2O$, H_2O to 1 L] in 1 L). Whilst submerged, the vitelline membrane encasing the yolk was cut circumferentially at the level of the equator and the membrane with embryo still attached transferred (outer surface down) to a watch glass. The membrane was stretched around a glass ring and any yolk attached to embryos carefully cleaned with a stream of PC saline. After conducting the experimental manipulation appropriate to each experiment (electroporation or bead grafts), the embryo on the vitelline membrane was transferred to a 35-mm Petri-dish containing a pool of albumen (Stern and Ireland, 1981) and allowed to develop in a 38°C humidified incubator.

Bead Grafting for Testing Secreted Factors

Embryos were put into New culture as described above. Prior to culture, beads appropriate to the factor tested (depending on protein charge and binding specificity: Heparin Acryllic, AG1X2 formate, or Affigel Blue beads)

were loaded by incubation with the protein for at least an hour, at a known loading concentration: 1000 μM somatostatin (Tocris), 5 μg/ml all-trans retinoic acid (Sigma), 2 μM Ionomycin (Sigma) (on AG1X2 beads); 2 μg/ml mouse recombinant Noggin (Sigma), 25 μg/ml mouse FGF-8b (Sigma) (Heparin Acryllic beads) and recombinant mouse Insulin-like Growth Factor II (Sigma) (loaded on Affigel blue beads). Once beads had been saturated with the signalling proteins they were rinsed briefly, in saline solution, and grafted to the extra-embryonic area of the embryo. After conducting the experimental manipulation appropriate to each experiment, the embryo on the vitelline membrane was transferred to a 35-mm Petri-dish containing a pool of albumen (Stern and Ireland, 1981) and allowed to develop in a 38°C humidified incubator for between 6 and 16 hours as appropriate to the experiment. Cultured embryos were fixed with 4% paraformaldehyde on the membrane and transferred to a plastic dish for overnight fixation at 4°C before being processed for whole mount in situ hybridisation.

Chick in vivo electroporations

Electroporation was carried out as previously described (Voiculescu et al., 2008). Embryos were removed from the vitelline membrane and transferred to an electroporation chamber in Tyrode's saline. The embryo was placed above the positive electrode ventral-side-up and a small volume of electroporation mixture containing a 2 – 2.5 μg/ml plasmid (constitutively active β-actin promoter driving the sequence encoding the protein of interest) in 6 % sucrose with 0.04% Fast Green FCS, was applied to the surface of the embryo with a capillary pipette. The negative electrode was positioned above the embryo and three pulses of 7.5 V, of 50 msec duration, 500 msecs apart, applied. The embryo was then rinsed with saline and replaced onto the vitelline membrane. Plasmids used included pcDNA3.1XSIP1venusC. Embryos were then placed in New culture and developed in a humidified chamber at 38°C.

Xenopus microinjections

Xenopus laevis embryos were obtained by in vitro fertilization, de-jellied in 2 % cysteine (Smith and Slack, 1983), cultured in 10 % Normal Amphibian Medium (NAM) (Slack et al., 1984) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Microinjections were carried out as previously published (Marchant et al., 1998) using capped mRNA transcribed from Smad6-pCS2+ (Yamada et al., 1999) and FGF8a-pCS2 (Fletcher et al., 2006) plasmids using mMessage mMachine (Ambion). The capped mRNA was injected into the animal pole of one cell at the two-cell stage or into blastomere A4 at the 32-cell stage together with 75 pg β-galactosidase and 5 ng lysine-fixable fluorescein dextran (FDX: Molecular Probes) as lineage tracers. Embryos were allowed to develop in 10% NAM to the stages indicated in the text, de-chorionated and fixed in MEMFA (4 % paraformaldehyde, 100 mM 3-(N-Morpholino)propanesulfonic acid [MOPS], pH 7.4, 2mM EGTA 1mM $MgSO_4$) at 4°C overnight.

Xenopus RNA-antisense probes.

The plasmids from which labelled RNA probes were made for in situ hybridization are listed in Table 2. Probes were synthesized from 3 μg of plasmid DNA, linearized using the appropriate restriction enzyme, before the antisense sequence was transcribed with either T7, T3 or Sp6 polymerase (Promega) in the presence of digoxigenin-labelled dUTP (Roche) to make a labelled anti-sense transcript.

Probe	Length	Marker For	Reference	Kind gift of
Brachyury	293	Mesoderm	(Smith et al., 1991)	J. Smith
Chordin	496	Mesoderm, notochord	(Sasai et al., 1994)	C. Hill
Myo-D	318	Mesoderm, notochord	(Hopwood et al., 1989)	N. Hopwood
Sox2	1400	Neural plate	(Kishi et al., 2000)	Y. Sasai
Sox3	1500	Neural plate	(Penzel et al., 1997)	R. Penzel
Neuronal-β-tubulin	1740	Primary neurons	(Oschwald et al., 1991)	K. Grunz

Table 2.2. Alphabetical list of *Xenopus* probes for in situ hybridization.

Xenopus whole-mount in situ hybridization.

Whole-mount in situ hybridisation was carried out as previously described (Marchant et al., 1998) through a three-day protocol. Fixed embryos were washed twice in PTW for 5 minutes before being dehydrated in 100% MeOH. Embryos were then progressively rehydrated in PTW before bleaching for 10 minutes (325 μl H_2O_2, 50 μl Formamide, 25 μl/ml 20x SSC) and re-fixing in 4% paraformaldehyde in PTW for 20 minutes. Embryos were then washed in 200 μl hybridization buffer (50 % formamide, 5x SSC, 1mg/ml Yeast RNA, 0.1% Tween, 10 nM EDTA, 0.1% CHAPS) at 62°C for 5 hours and incubated overnight (12 hours) with an RNA anti-sense probe on a 62°C heat-block. The following day vials were washed through a series of five 62 °C heated solutions for 10 minutes each (Solution 1: 50% Formamide, 2x SSC, 0.1% Tween, Solution 2: 25% Formamide, 2x SSC, 0.1% Tween. Solution 3: 12.5% Formamide, 2x SSC, 0.1% Tween). Three washes of 5 minutes of PTW and two washes of 5 minutes with TBST at room temperature were followed by blocking for 2 hours (5% heat inactivated goat serum and 1mg/ml bovine serum albumin in TBST). Embryos were then incubated overnight at 4°C in 1:5000 anti-digoxigenin alkaline phosphatase (Roche) in blocking buffer. Next, the embryos were washed through a series of TBST washes (6 washes for 45

minutes each) at room temperature. The embryos were then washed twice in alkaline phosphatase buffer (APB: 100 mM Tris Base pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween) for 10 minutes before the alkaline phosphatase signal was developed (3.5 μl BCIP, 4.5 μl NBT per ml of APB; see above). Once the colour had developed as desired, the β-galactosidase lineage-marker was visualised. First, embryos were briefly re-fixed in 1 x MEMFA for 10 minutes at room temperature; the embryos were then transferred into β-galactosidase staining solution (250 μl Solution A [0.2 M Phosphate buffer, pH 7.5, 20 mM KCl, 2mM $MgCl_2$], 50 μl Solution B [0.5 M $K_4Fe(CN)_6$], 50 μl Solution C [0.5 M K_3FeCN_6], 20 μl 40% X-gal in N-dimethylformamide, 50 μl Triton x100, 50 μl H_2O) at room temperature until the staining was apparent. Finally, stained embryos were rinsed twice in 1x PBS and fixed in 1x MEMFA.

9N2 Embryonic Stem cell culture and differentiation

Chick blastoderm derived stem cells line 9N2 (Petitte and Yaag, 1994) were maintained on a feeder layer of Sto-cells (mouse fibroblast cell line) in the presence of BRL conditioned medium. The feeder layer was prepared by mitotically inactivating Sto cells to prevent replication by treatment with 100 μg/ml mitomycin (Sigma) in PBS for 90 minutes, and plated at 1 x 10^5 density per well in 6 well tissue culture plates which had been pre-treated with 0.1% gelatine (Speciality Media, USA). An aliquot of 9N2 cells was thawed from liquid-Nitrogen storage and plated on the Sto feeder cells and expanded in 50% BRL conditioned medium. Conditioned medium was obtained from the supernatant of buffalo rat line (BRL) cells grown to confluence on 10 cm Petri-dishes in DMEM (10% Fetal Calf Serum, 2 μM Glutamine), and their supernatant harvested every three days over a nine day period. The supernatant was combined with other factors to make Complete Medium (28 mls BRL supernatant, 2.5 ml Fetal Bovine Serum, 50 μl β-mecaptoethanol, 500 μl nucleosides (x100), 500 μl sodium pyruvate, 500 μl non-essential amino acids (x100), 500 μl glutamine, 500 μl Penicillin-Streptomycin, 15 mls Knock-out DMEM) which was then mixed 50:50 with DMEM (10 % Fetal Calf

Serum, 2 mM Glutamax). Once the 9N2 cells had been sufficiently expanded they were plated at low density (30 % confluency) into 24-well tissue culture plates with feeder layer on polylysine-treated coverslips, allowed to settle overnight and a modified differentiation procedure initiated. A neural differentiation protocol was adapted from previously published mouse ES protocols (Lee et al., 2000; Ying et al., 2003b); initial treatment involved 1 day in ADFNK medium (21.5 ml neurobasal medium, 21.5 ml knockout DMEM, 5 ml Fetal Calf Serum, 500 μl Pen-Strep, 500 μl Glutamax, 400 μl β-mercaptoethanol, 500 μl N2 supplement, 500 μl B27 supplement) followed by 4 days with Retinoic Acid (DMEM 10% FCS, 2 μM Glutamine, 1 x 10^{-6} M Retinoic Acid). A range of treatments and lengths of culture time were then attempted depending on the experiment. Treatments were as follows: 4 days of growth factors: mFGF8b 100 ng/ml, Sonic Hedgehog 400 ng/ml, GDNF 5 ng/ml. followed by 13 days in N2 B27 supplemented DMEM. Additional factors used included the Wnt signalling inhibitor Dkk (100 ng/ml) and Wnt3a treatment (25 ng/ml) as described in the text. Once cells had been grown to the desired time point they were fixed overnight at 4°C before being processed for *in situ* hybridization or immunohistochemistry.

In situ hybridisation of cultured 9N2 ES cells

The differentiated 9N2 cells were retained in their 24-well tissue culture plates (still adhered to the polylysine treated coverslips), and fixed in 4 % paraformaldehyde overnight at 4°C, then placed overnight in absolute methanol at -20°C. The following day cells were progressively rehydrated through 5 min washes in 75%, 50% and 25% methanol:PTW and finally into PTW for two 5 minute washes. Cells were post-fixed for 30 min in 4% PFA 2 mM EGTA 0.1 % glutaraldehyde in PBS) and finally into hybridization solution and incubated at 68°C for 2 hours before incubation overnight with DIG-labelled riboprobe for the desired gene. Day 2 and Day 3 progressed as for chick whole mount in situ hybridization protocols (see above).

Immunohistochemisty of cultured 9N2 ES cells

Cells were washed for 3 x 5 min with PBS before incubating for 30 min with blocking solution (PBS + 1% Bovine Serum + 0.1% Triton X100). Cells were incubated overnight in primary antibody at 4°C. Primary antibodies used were as follows: Transitin A2B11 (rat IgM), Pax6 (mouse IgG1), 3A10 (mouse IgG1) (these antibodies developed by G.J. Cole, A. Kawakami, and T.M. Jessell respectively, were obtained from the Developmental Hybridoma bank developed under the auspicies of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA52242) δ-crystallin (sheep) (a gift from J. Piatigorsky). The following day cells were washed three times 5 min with PBS and incubated for 1 hour with the appropriate secondary antibody (Alexa-594 goat anti-rat IgM, Alexa-488 donkey anti-mouse IgG or Alexa-488 donkey anti-sheep IgG). Finally the cells were washed for 3 x 5 minutes with 1xPBS then incubate for 15 minutes in 1ug.ml DAPI (4'-6-dmamidine-2'-pjenylindole dihydrocholoride) solution (Roche) at room temperature before being washed in 100% methanol. Finally, coverslips were inverted and mounted onto standard slides and fluorescence visualized by compound microscopy.

Chapter Three

Characterisation and regulation of genes induced in response to neural induction signals from the organizer

Introduction

In the chick at least 12 hours' exposure to signals from the node are required for cells to express the neural plate marker *Sox2* and to progress to form an ectopic neural plate (Gallera and Ivanov, 1964; Gallera, 1970). BMP inhibitors are unable to replicate the effect of a grafted node as no neural markers are up-regulated in response to a source of any combination of BMP inhibitors (Streit and Stern, 1999; Streit et al., 2000; Linker and Stern, 2004). However, if the area opaca cells are exposed to signals from a grafted node for a minimum of 5 hours followed by exposure to a source of BMP inhibitors once the node is removed, expression of the early neural marker *Sox3* is maintained (Streit et al., 1998), suggesting that at least 5 hours of signals from the node are required to sensitize cells to BMP signalling.

These results raise the question: What is different between cells that have or have not been exposed to signals from the organizer for 5 hours? In other words, what is different between cells that have or have not been sensitised to BMP signals? To understand the differences between these two conditions, a differential screen was carried out to identify genes upregulated in response to 5 hours of signals from Hensen's node (Streit et al., 2000). This screen compared the expression of genes in area opaca epiblast cells that had been exposed to 5 hours of signals from a grafted Hensen's node with area opaca epiblast from the contralateral side of the embryo which had received no treatment (Streit et al., 2000). In total, 10 genes were identified that differ between the two conditions. Eight of these; *ERNI, Churchill, Calfacilitin, Dad1,*

polyubiquitin, and ferritin heavy chain, *Asterix* and *Obelix* have been described to date (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010; Pinho et al., 2011) (Papanayotou unpublished observations). Importantly, these genes are all expressed in prospective neural tissue during development; furthermore, they can all be induced by a source of FGF8 and their induction by a grafted node is lost if FGF signalling is blocked (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010). However, despite this evidence suggesting that FGF signalling appears to be the signal that mediates the early effects of the node, even a combination of FGFs and BMP inhibitors is insufficient to induce the neural marker *Sox2* in the area opaca, suggesting additional signals must be involved (Linker and Stern, 2004).

Another gene that in the neural induction cascade is Bert, which was initially identified through a yest-2-hybrid screen for binding partners of the early response gene, *ERNI* (Papanayotou et al., 2008). Bert has an important function in the neural induction cascade acting on an epigenetic regulatory switch to initiate the expression of the pan neural marker *Sox2* in the neural plate (Papanayotou et al., 2008). It is expressed after 11-12 hours of signals from a grafted node, but is not induced by FGF, or even by FGF together with BMP inhibitors, suggesting that additional signals must be involved

There are three remaining genes from the 5 hour response screen that remain to be fully characterized. Two of them are novel genes, not previously described, and the remaining gene *TrkC*, known to encode a neurotrophin receptor (Bernd and Li, 1999), has not previously been considered in the context of neural induction. Thus, it is important to characterize the remaining genes, validate the results from the screen by establishing if they are expressed in prospective neural cells during normal development and identify their place in the neural induction response cascade. This chapter aims to identify the signals that both these early response genes and the later neural marker *Bert*.

Methods

The differential screen for early responses to signals from a grafted of Hensen's node was carried out as previously described (Streit et al., 2000) and identified two novel transcripts given the names *Asterix* and *Obelix*. These were characterized by S. Pinho and P. Simonsson (Pinho et al., 2011). Neural induction assays were performed as described in Chapter 2; FGFs were delivered using Heparin acrylic beads (Sigma) soaked for 2 hours in 5 μl mouse recombinant FGF-8b (0.1 μg/ml, R&D). SU5402 was delivered using AG1X2 ion exchange beads (Formate form) soaked in 25 μM SU5402 (Calbiochem).

Work in this chapter was carried out in collaboration with P. Simonsson, S. Pinho, W. Sherlock, and K. Trevers where noted. Fuller descriptions of methods for molecular characterisation have been published in Pinho et al., 2011 (Pinho et al., 2011).

Results

Characterisation of *Obelix* (these experiments were conducted by S. Pinho) and *Asterix* (these experiments were done by P. Simonsson)

Two transcripts isolated in a differential screen for genes up-regulated in response to 5 hours of signalling from a grafted node were found to encode novel proteins. The first of these, *Obelix*, was found to encode a domain similar to the olignucelotide /oligosaccharide-binding (OB) domains of the Translation Initiation Factor eIF1A (TFeIF1A) as determined by BLAST analysis (Fig. 3.1 A). The OB domain of TFeIF1A is responsible for the RNA-binding properties of the protein (Battiste et al., 2000). Because of this, and its predicted globular shape, the protein was designated *Obelix* (GenBank accession number AY103477). Phylogenetic bootstrap analysis using SeaView 4.2.12 (Gouy et al., 2010) reveals that *Obelix* is conserved across phyla and encodes a family similar but distinct from eIF1A, as its predicted

structure contains an extra-sheet structure between sheets β3 and β4 rather than a helix (Battiste et al., 2000).
To gain insight into the intracellular location of *Obelix*, a myc-tagged version was transfected into COS-1 cells, and the presence of *Obelix* protein in cell lysates and medium was assayed by Western blotting. Obelix protein was detected in the cell lysate (C, Fig. 3.2. A), but not in the supernatant (S, Fig. 3. 2 A), suggesting a cellular protein that is not secreted. Immunostaining of transfected COS-1 cells or transfected chick embryo epiblast revealed a predominantly nuclear localization (Fig. 3.2. B-D).

The second transcript isolated from the screen encodes a predicted protein product belonging to an Uncharacterized Protein Family designated UPF0139, (also designated CGI140 or c19orf56 in humans because the predicted open reading frame appears on chromosome 19) which is very highly conserved across all vertebrates as well as invertebrates (Fig. 3.3). Because of the smaller size of the predicted protein as compared to *Obelix,* and their co-expression, it was designated as *Asterix* (Genbank accession number HQ184923).

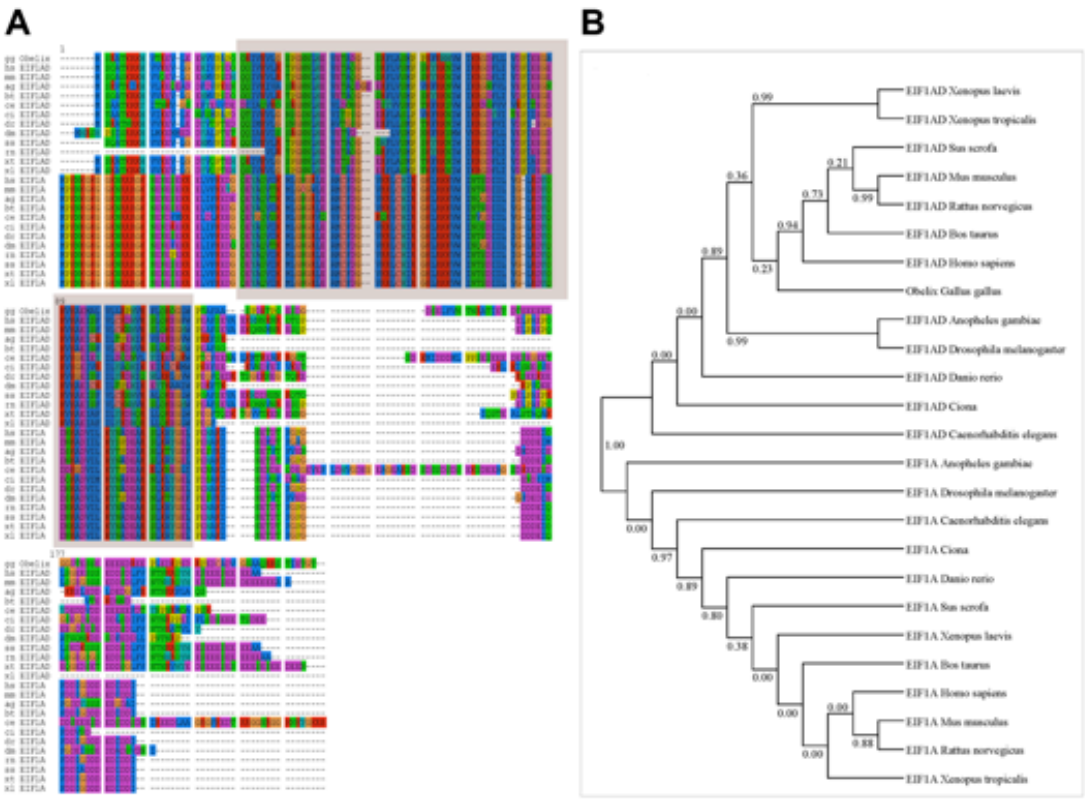

Fig. 3.1. Molecular characterization of *Obelix*. A. Sequence alignment of *Obelix* protein (AY103477) with ESTs for EIF1A-related proteins from several species. Residues are displayed in different colours based on different amino acid families and degree of homology is represented by conservation of these sites. Conserved OB-like domain is shown as a block in the alignment. Species are abbreviated as follows: ag, *Anopheles* mosquito (BM594550); bt, cow (BF043073); ce, *C. elegans* (AV203381); ci, *Ciona* (AV841463); dm, *Drosophila melanogaster* (BE977318); dr, zebrafish (BM859434); hs, human (BG149615); mm, mouse (BI103120); ss, pig (BG610103); rn, rat (BF420639); xl, *Xenopus laevis* (BG730245); xt, *Xenopus tropicalis* (AL637659). **B.** Phylogenetic tree with bootstrap values comparing the full-length sequences of *Obelix* in a variety of species, showing that eIF1A and *Obelix* segregate into two distinct sub-classes of OB-containing proteins. The LG model was used to construct the tree and bootstrap values were calculated from 1000 replicates.

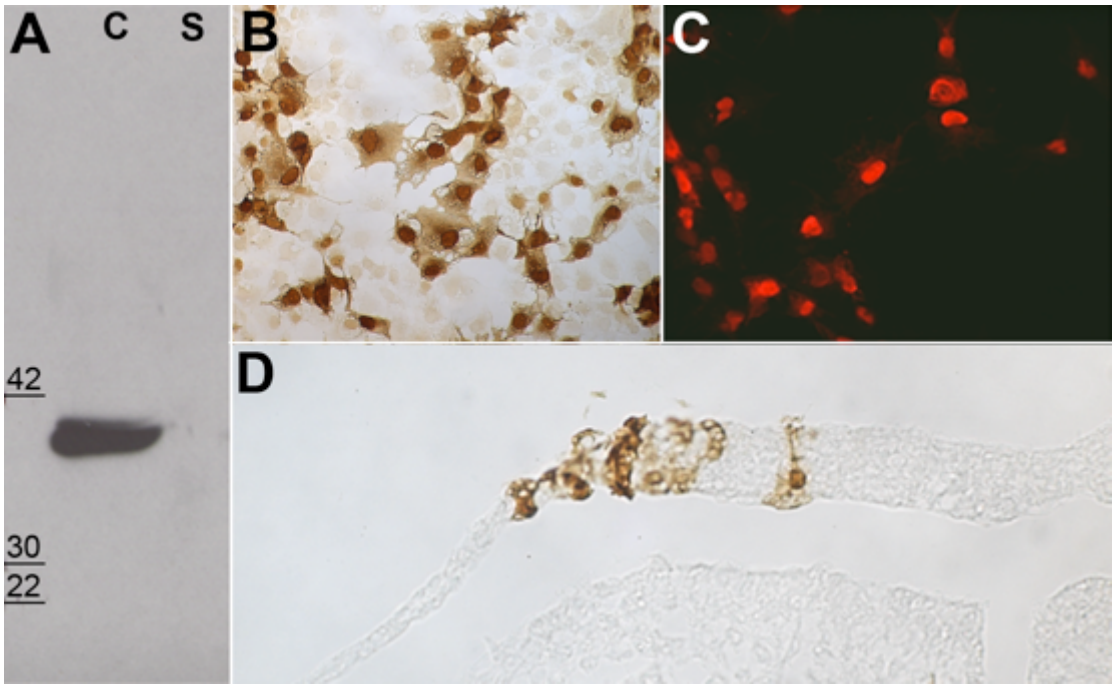

Fig. 3.2. *Obelix* is intracellular and localizes to the nucleus. A. *Obelix* protein can be retrieved from cell extracts (C) but not from the supernatant (S) of transfected COS-1 cells, and detected by Western blotting. B-D. Nuclear localization of Myc-tagged *Obelix* protein can be seen in transfected COS-1 cells (B, C) as well as in the neural plate of a chick embryo (D). In B and D the anti-Myc antibody is revealed by peroxidase staining with diaminobenzidine; in C the signal is revealed with Cy3-coupled anti-mouse antibody.

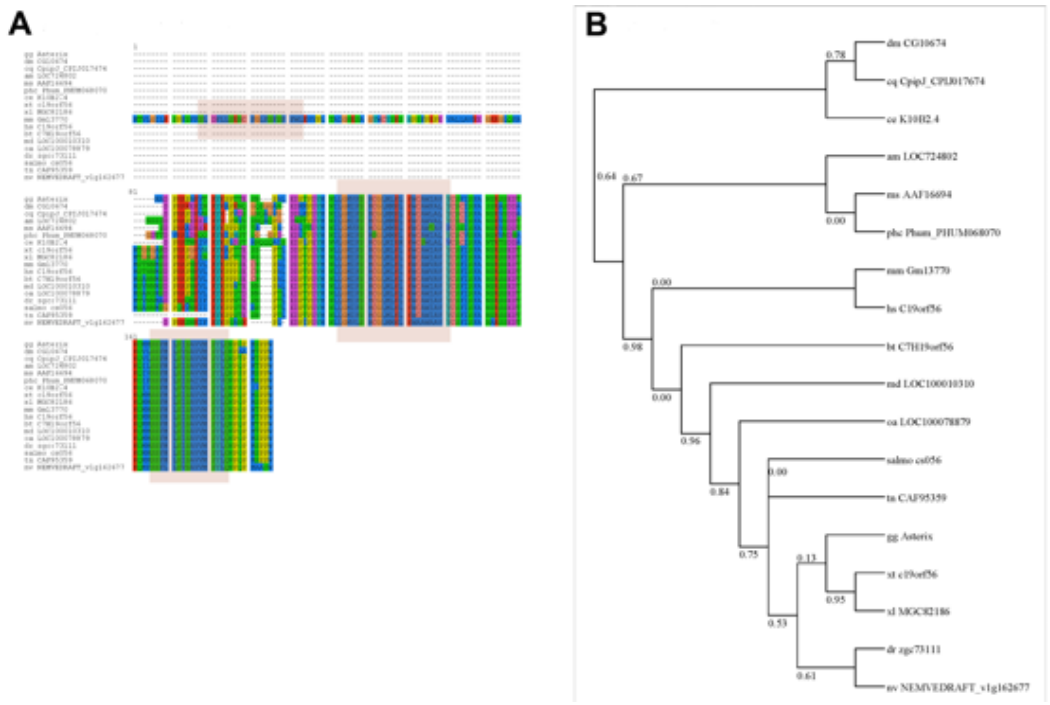

Fig. 3.3. Molecular characterisation of *Asterix*. A. Sequence alignment of *Asterix* protein with related proteins in other species. Residues are displayed in different colours based on different amino acid families and degree of homology is represented by conservation of these sites. Predicted transmembrane domains are outlined as blocks. Abbreviations for the following organisms appear in the alignment in the following order from top to bottom: Chick (gg) *Asterix*; *Drosophila melanogaster* (dm) protein (CG10674); *Culex quinquefasciatus* (cq) hypothetical protein (CpipJ_CPIJ017674); *Apis mellifera* (am) protein (LOC724802); *Manduca sexta* (ms) unknown protein (AAF16694); *Pediculus humanus corporis* (phc) hypothetical protein (Phum_PHUM068070); *Caenorhabditis elegans* (ce) hypothetical protein (K10B2.4); *Xenopus tropicalis* (xt) protein (c19orf56); *Xenopus* laevis (xl) protein (MGC82186); *Mus musculus* (mm) predicted gene 2573 (Gm13770); *Homo sapiens* (hs) protein (c19orf56); *Bos taurus* (bt) c19orf56 ortholog (C7H19orf56); *Monodelphis domestica* (md) similar to CGI-140 protein (LOC100010310); *Ornithorhynchus anatinus* (oa) hypothetical protein (LOC100078879); *Danio rerio* (dr) hypothetical protein (zgc:73111); Salmo *salar* (salmo) UPF0139 membrane protein C19orf56 homolog (cs056); *Tetraodon nigroviridis* (tn) unnamed protein (CAF95359); *Nematostella vectensis* (nv) hypothetical protein (NEMVEDRAFT_v1g162677). B.

Phylogenetic tree with bootstrap values comparing the full-length sequence of *Asterix* with homologues from other species. The LG model was used to construct the tree and bootstrap values were calculated from 1000 replicates.

Expression of *Obelix* and *Asterix* during development

(Performed in collaboration with S. Pinho and P. Simonsson)

Both *Obelix* and *Asterix* were identified from a screen for early responses to signals from a grafted organizer (Hensen's node). If these genes are indeed responses to neural induction, they should also be expressed in the early neural plate of normal embryos at appropriate stages. To test this, whole mount in situ hybridization was performed on early chick embryos. *Obelix* transcripts are first detected at the mid- to late-primitive-streak stage (stage 3^{+}), initially in a region of the area pellucida a little broader than the future neural plate (Fig. 3.4. A, E). Expression quickly becomes confined to the neural plate (Fig. 3.4. B, F) where it remains until at least stage 14, including streams of neural crest cells migrating away from the neural tube (Fig. 3.4. D).

Asterix expression is first detected very weakly in the hypoblast and Koller's sickle at pre-primitive streak stages (Eyal-Giladi and Kochav, 1976); stages XI-XIII; Fig. 3.5. A-B). During primitive streak formation it is expressed in the streak itself (Fig. 3.5. C). From stage 4 (Fig. 3.5 D, Fig. 3.6 A, B) expression is seen in the node, the lips of the streak and the epiblast in the middle of the area pellucida but is absent from more peripheral regions (future epidermis and extraembryonic ectoderm). By the start of neurulation (stage 7) expression becomes progressively concentrated in the neural plate (Fig. 3.5. E-H, Fig. 3.6. C-I), neural tube (Fig. 3.5. I-K, Fig. 3.6 J-M) and sensory placodes including lens, otic and olfactory placodes (Fig. 3.5. J-M, Fig. 3.6. K-N). From stage 16 expression starts to decrease in the nervous system to become concentrated mainly in the notochord (Fig. 3.5. M, Fig. 3.6. O-Q), as well as remaining in the sensory placodes. Some expression is also seen in somites (e.g. Fig. 3.5. H, Fig. 3.6 I) and persists in the myotome at later stages (Fig. 3.6. P).

In conclusion, both *Obelix* and *Asterix* are expressed in the developing nervous system. They appear in the prospective neural plate during

gastrulation (stage 3^+) and remain expressed in the neural plate, neural tube, neural crest (*Obelix*) and placodes (*Asterix*) until stage 14.

TrkC expression during development

(Performed in collaboration with K. Trevers)

A third gene identified by the screen for early responses to a grafted node encodes the neurotrophin receptor, *TrkC*. Its expression has previously been described during quail development but not in sufficient detail to determine a precise time course during neural induction (Yao et al., 1994; Zhang et al., 1994; Zhang et al., 1996; Bernd and Li, 1999). We therefore studied its expression by in situ hybridization in chick embryos between pre-streak and neural plate stages (Fig. 3.7. A-H). Transcripts are first detected close to Hensen's node at stage 3^+ (Fig. 3.7. C-D) from where expression expands to the forming neural plate between stages 4-7 (Fig. 3.7. E-G). Thereafter it remains expressed almost throughout the neural plate except in the most caudal domains and in sub-regions of the hindbrain (Fig. 3.7. G, H) (see also (Zhang et al., 1996; Bernd and Li, 1999)).

Time-course of induction of *TrkC, Obelix* and *Asterix* by the organizer

(Performed in collaboration with K. Trevers, S. Pinho and P. Simonsson)

To confirm that *TrkC*, *Obelix* and *Asterix* are indeed early response genes to signals from the organizer, their induction by Hensen's node grafts was studied in time-course. No induction is seen 2 hours after a node graft into the area opaca of HH3+/4 host embryos (*Obelix*: 0/4; Fig. 3.8. A, D; *Asterix:* 0/6; Fig. 3.9. A, E). At 3 hours, *Obelix* and *TrkC* are very weakly induced in a minority of embryos (*Obelix*: 4/13, Fig. 8 B, E; *TrkC*: 6/8 Fig. 3.7. I) and *Asterix* not at all (0/7). A short time later, all genes are strongly induced: *Obelix* induction appears 5 hours after the node graft (18/19; Fig. 3.8. C, F), and *Asterix* and *TrkC* by 4-5 hours (*TrkC*: 4 hours: 3/3; 5 hours: 3/3; 6 hours: 5/5; Fig. 3.7. J, K; *Asterix:* 4 hours: 7/9; 5 hours: 7/8; Fig. 3.9. B, F). Together

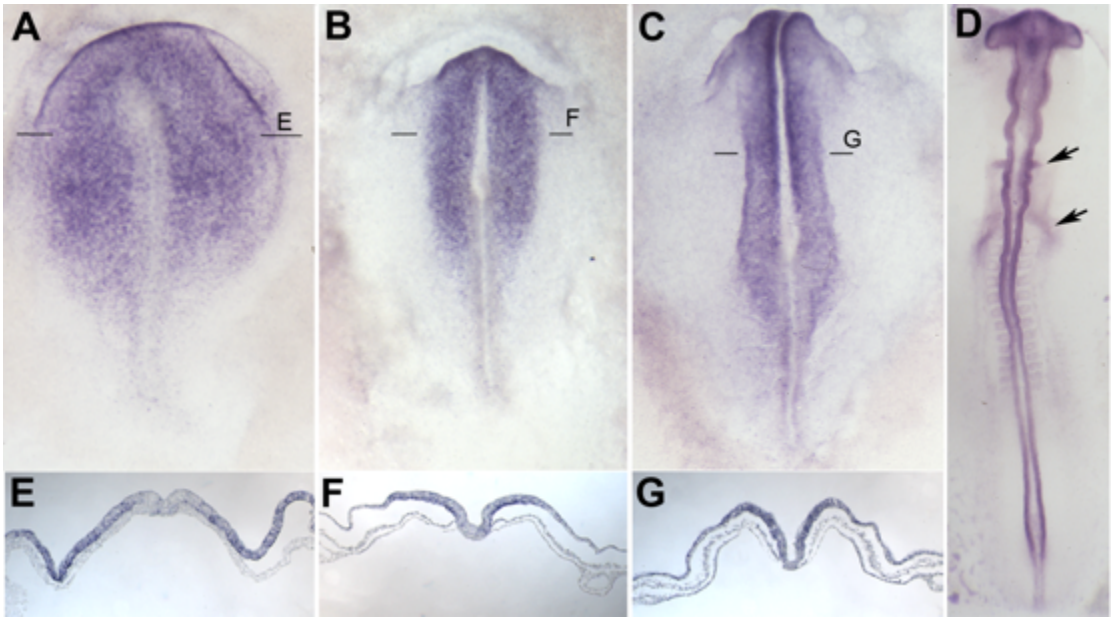

Fig. 3.4. *Obelix* expression during early development. Expression of *Obelix* by in situ hybridization at stages 3^{+} (A), 5 (B), 7 (C) and 11 (D). E-G are sections through the levels shown in A-C. Expression is localized in the neural plate, neural tube and their derivatives.

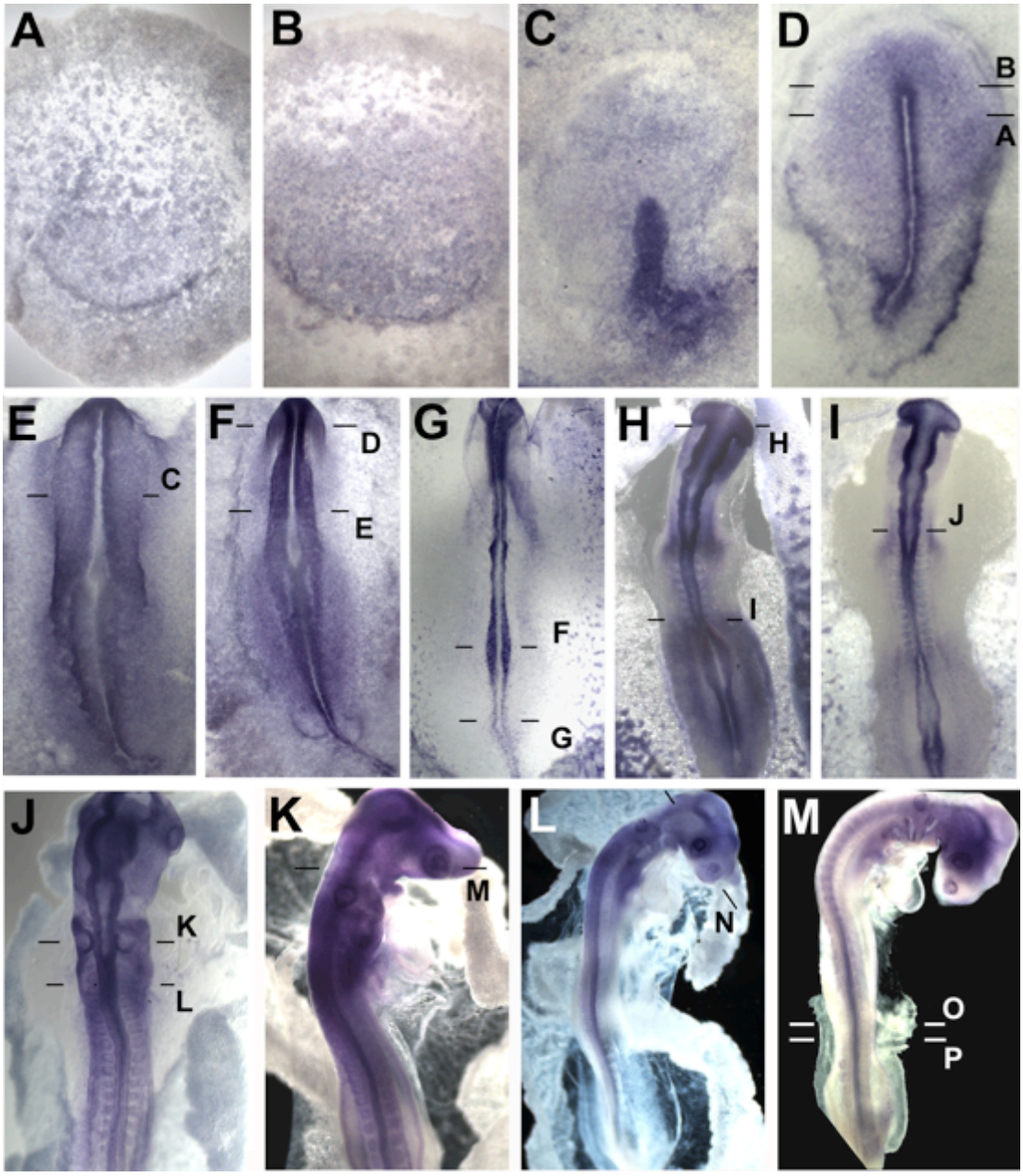

Fig. 3.5. Expression of *Asterix* during development. Embryos at stages XI (A), XII (B), 3 (C), 4+ (D), 6 (E), 7 (F), 9 (G), 10 (H), 11 (I), 14 (J), 16 (K), 17 (L) and 18 (M) are shown. The horizontal lines and letters refer to the levels at which sections in Fig. 3.6 were taken.

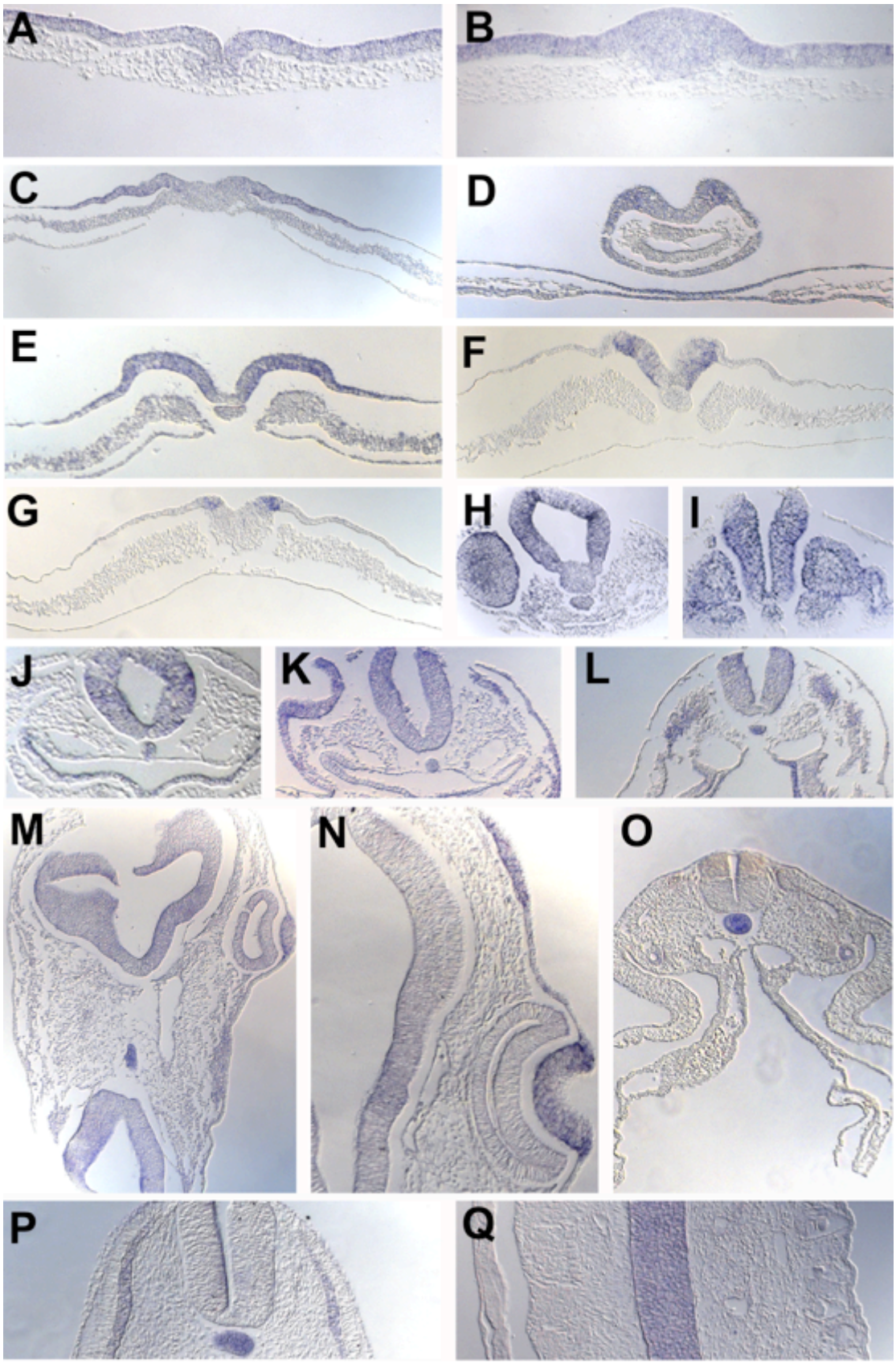

Fig. 3.6. Expression of *Asterix* during development (continued).

Fig. 3.6. Expression of *Asterix* during development (continued)**.** Sections through embryos at stages 4+-18, at the levels indicated in Fig. 3.5 Q shows a coronal section through an embryo at stage 16, showing expression in the notochord.

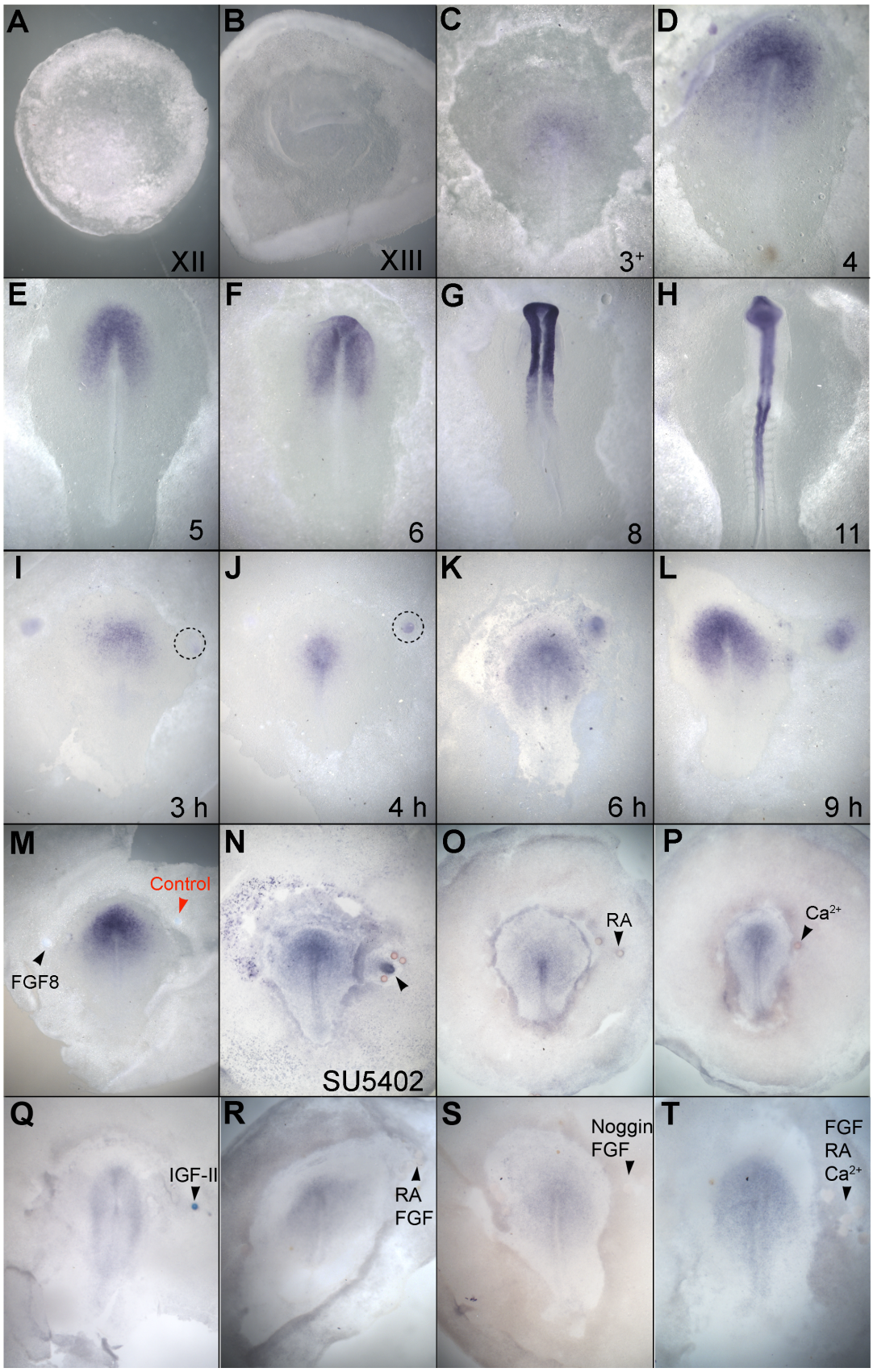

Fig. 3.7. Expression and regulation of *TrkC*.

Fig. 3.7. Expression and regulation of *TrkC*. A-H. *TrkC* expression during early chick development. The stage of development is indicated on the lower right of each panel. Expression begins at mid-primitive streak stage (stage 3^+; C) and intensifies in the neural plate thereafter (E-H). **I-L.** Time-course of induction of *TrkC* by a graft of Hensen's node. Induction begins about 4 hours after grafting (J) and becomes strong after about 6 hours (K, L). **M.** *TrkC* is not induced by FGF. Embryo shown 6 hours after implantation of a bead soaked in FGF8b (black arrow) and a control bead (red arrow). **N.** Induction of *TrkC* by the organizer does not require FGF signals. Embryo shown 6 hours after co-transplantantation of a Hensen's node and three beads soaked in SU5402. Induction of *TrkC* is not inhibited (arrow). **O.** *TrkC* is not induced by retinoic acid (arrow). **P.** *TrkC* is not induced by a bead soaked in ionomycin to increase intracellular Calcium (Ca^{2+}; arrow) **Q.** *TrkC* is not induced by insulin-like growth factor-II R, *TrkC* is not induce by a combination of retinoic acid and FGF soaked beads (arrow), **S.** *TrkC* is not induced by a combination of Noggin and FGF (arrow), **T.** *TrkC* is not induced by a combination of three beads soaked in FGF, retinoic acid and ionomycin to increase intracellular calcium (arrow).

with the findings that all three genes are normally expressed in the prospective and early neural plate, these findings confirm *TrkC*, *Asterix* and *Obelix* as early responses to neural inducing signals from the organizer, Hensen's node.

Regulation of *Asterix*, *Obelix*, *TrkC* by secreted factors

(Performed in collaboration with P. Simonsson, S. Pinho, K. Trevers and W. Sherlock).

Next, we sought to determine whether any known secreted factors implicated in neural induction or expressed in the organizer can mimic the ability of the node to induce *Asterix*. Unlike BMP- and Wnt-inhibitors, FGF8-soaked heparin beads induce *Asterix* expression in the adjacent epiblast within 5 hours (2-3 h: 2/16; 4-5h: 12/15; 16-18h: 7/9; Fig. 3.9. C, G) without inducing the mesodermal marker *Brachyury* (0/10; Fig. 9 D, H). To test whether FGF signalling from the organizer is required for *Asterix* induction, a node was transplanted together with beads soaked in SU5402, an inhibitor of the FGF receptor (Mohammadi et al., 1997), into the extraembryonic region. This completely abolishes *Asterix* induction (0/8; Fig. 3.9. I), unlike control DMSO-soaked beads (4/4; Fig. 3.9. J). SU5402 also inhibits induction of *Sox3* by a grafted node (0/6; Fig. 3.9 K, 4/4 control, Fig. 3.9. L), as previously described (Streit et al., 2000). In conclusion, FGF8 mimics the ability of the node to induce *Asterix* within 5 hours and FGF activity is necessary for its induction by the node.

Rather different results are obtained for *TrkC* and *Obelix*. FGF8 does not induce *TrkC* expression at all (0/11 after 6 hours, 0/12 after 14 hours; Fig. 3.7. M). FGF4 or FGF8-coated beads do induce *Obelix* (6/14 and 11/15, respectively; Fig. 3.10. B, E, M, N). However, in contrast with Hensen's node grafts, induction by either factor is weak, localized to the immediate vicinity of the bead and only seen in a subset of embryos (17/29, 58%). We also tested many other candidate factors, none of which induces either gene: retinoic acid

(*Obelix:* 0/11; Fig. 3.10. J: *TrkC*: 0/4, Fig. 3.7. O), ionomycin to increase intracellular Calcium (*Obelix*: 0/9 Fig. 3.10. I; *TrkC*: 0/4, Fig. 3.7. P), Chordin (*Obelix*: 0/8; Fig. 3.10. C), Noggin (*Obelix*: 0/6; Fig. 3.10. D, P), the Wnt antagonist Dkk1 (*Obelix*: 0/10; Fig. 3.10. F, R), Cerberus (*Obelix*: 0/12; Fig. 3.10. G, S), HGF/SF (*Obelix*: 0/10; Fig. 3.10. H, T), Somatostatin (*TrkC*: 0/8; *Obelix*: 0/15; Fig 3.10. K), insulin-like growth factor-II (*TrkC:* 0/6 Fig 3.7. Q; *Obelix*: 0/9 Fig. 3.10 L), nor FGF in combination with retinoic acid (*TrkC* 0/5 Fig 3.7. R), noggin (*TrkC* 0/7 Fig 3.7. S), or retinoic acid and ionomycin (*TrkC* 0/4 Fig 3.7. T).

These results suggest that FGFs induce *Obelix*, but only weakly and not in all cases and that *TrkC* is not induced by FGF at all. To test whether FGF signalling from the node is required for *TrkC* and *Obelix* induction, we transplanted the organizer together with SU5402-coated beads. Both *TrkC* (6/6; Fig. 3.7. N) and *Obelix* (4/5; Fig. 3.10. T-Z) are induced even when FGF signalling is inhibited. This is in contrast to *ERNI, Sox3*, *Churchill* and *Sox2* induction, all of which require FGF activity in the same assay (Streit et al., 2000; Sheng et al., 2003).

In conclusion, the three early response genes differ in the extent to which they are inducible by FGF: FGF is both necessary and sufficient to induce *Asterix*, sufficient but not necessary to induce *Obelix* and neither sufficient nor necessary for induction of *TrkC*. A plausible interpretation is that *Obelix* may be induced by a factor other than FGF which acts through the same pathway(s), such as PDGF (platelet derived growth factor), whereas *TrkC* is likely to be induced by factor(s) acting through other pathways.

Regulation of *Bert* by secreted factors

Unlike the other genes investigated here, *Bert* is expressed later in the neural induction cascade, requiring 11-12 hours of exposure to the node (Papanayotou et al., 2008). Previous studies have shown that it cannot be induced by a source of FGF, BMP antagonists or Wnt inhibitors (Papanayotou

et al., 2008). Thus, we sought to determine whether any known secreted factors implicated in neural induction or expressed in the organizer can induce the expression of *Bert*.

We tested many factors, none of which either independently or in combination were able to induce the expression of *Bert* including, retinoic acid (0/8 Fig. 3.11. B), ionomycin to increase intracellular calcium (0/4 Fig. 11 E), somatostatin (0/7 Fig. 3.11. F), insulin-like growth factor-II (0/7 Fig 3.11. D), a high concentration of noggin (0/7 Fig. 11 G), FGF with retinoic acid (0/5 Fig. 3.11. C) FGF with noggin (0/4 Fig. 3.11. H). Furthermore, the combination of FGF with noggin was also unable to induce the neural plate marker *Sox2* (0/6 Fig. 3.11. I).

In conclusion, no single factor, or combination of factors yet tested is sufficient to induce *Bert*. The finding that even a high level of BMP antagonists alone or in combination with FGF are insufficient to induce *Bert* or the neural plate marker *Sox2* suggests that the level of BMP inhibition alone cannot explain differences in requirement of BMP inhibition between chick and *Xenopus* experiments.

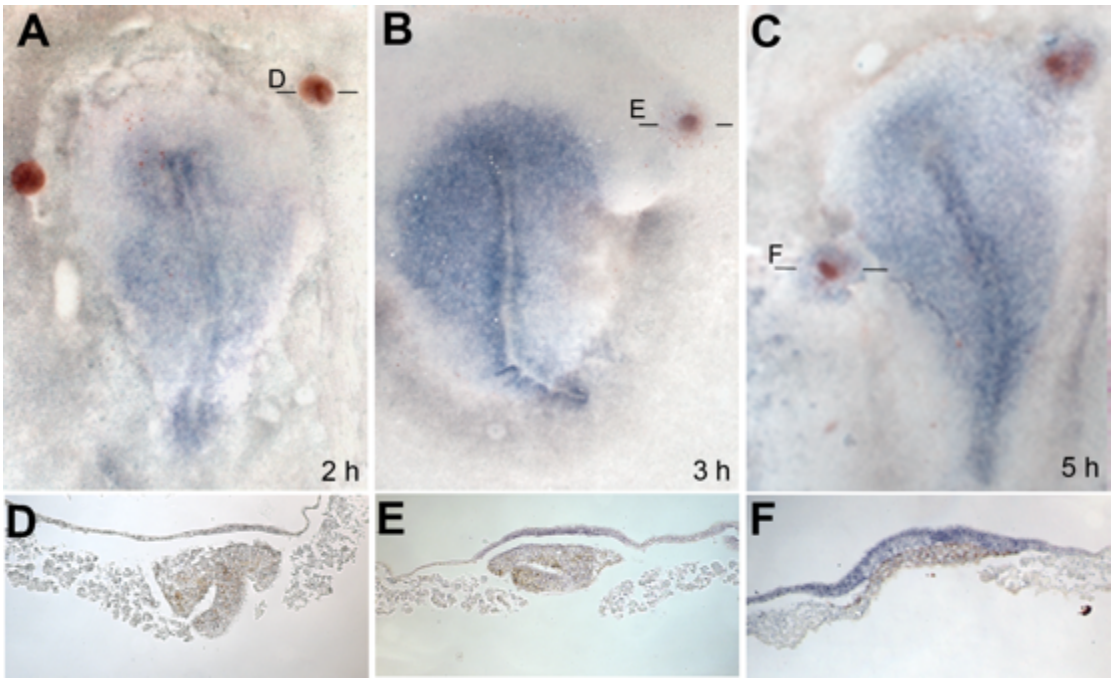

Fig. 3.8. Time-course of induction of *Obelix* by Hensen's node. Time-course of induction by grafts of a quail node into a chick host. No induction is seen at 2 hours (A), weak induction starts at 3 hours (B) and robust induction is seen by 5 hours (C). D-F are sections through the grafted regions of the embryos in A-C at the levels indicated. Quail donor cells are stained brick-red by QCPN antibody and *Obelix* mRNA in purple/blue.

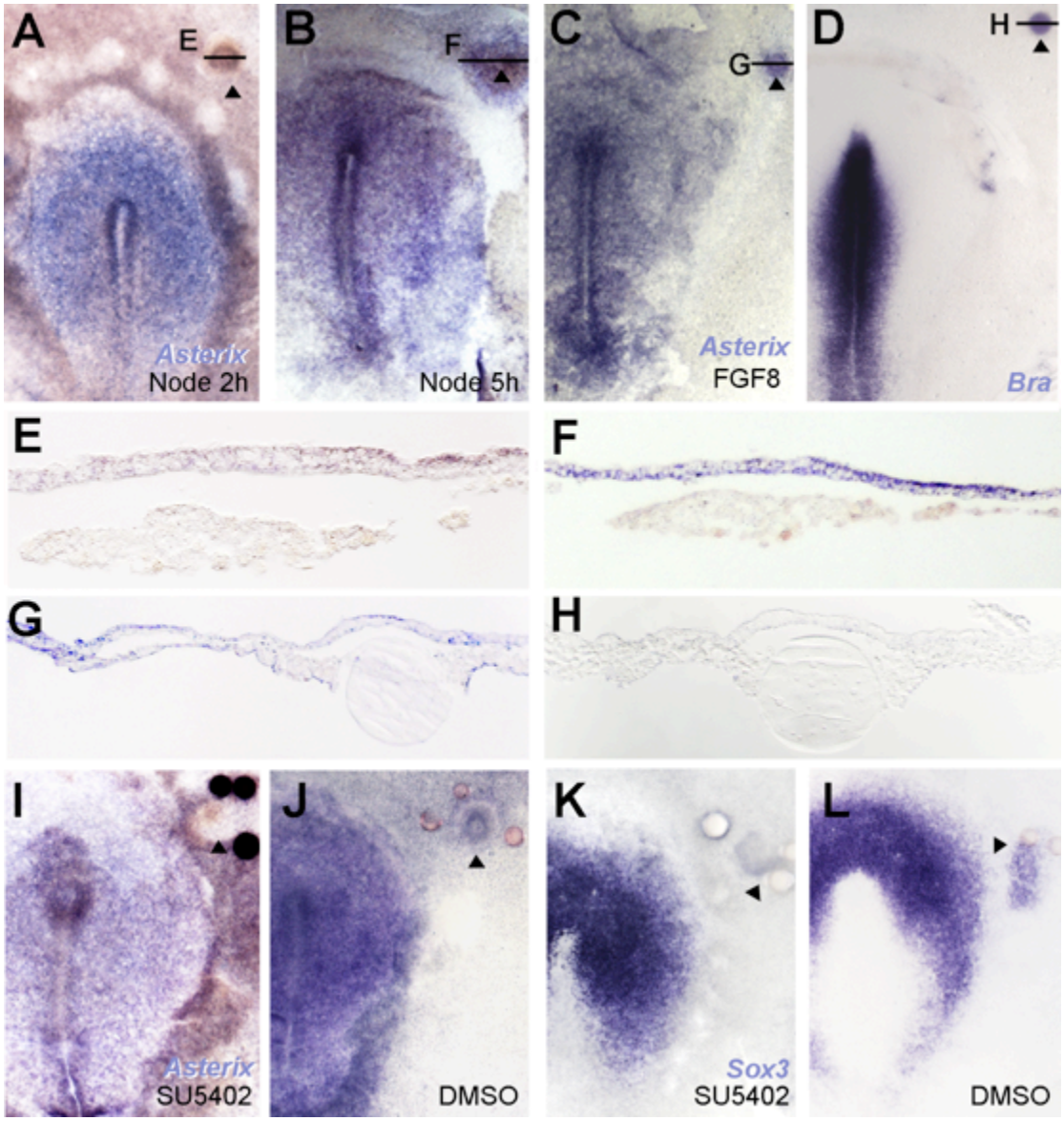

Fig. 3.9. Regulation of *Asterix* by Hensen's node and peptide factors. Grafts of Hensen's node do not induce *Asterix* after 2 hours (A, E), but do induce it after 5 hours (B, F). This is mimicked by grafts of heparin beads soaked in FGF8, which induce *Asterix* (C, G) but not *Brachyury* (D, H). When a node is grafted together with beads soaked in the FGF inhibitor SU5402, *Asterix* induction is blocked (I), as is induction of the early pre-neural marker *Sox3* (K). Grafts of the node together with the vehicle DMSO do not affect induction of either marker (J for *Asterix*, L for *Sox3*). Note that some probes attach non-specifically to some types of beads and to COS cell pellets (eg. panels H, I).

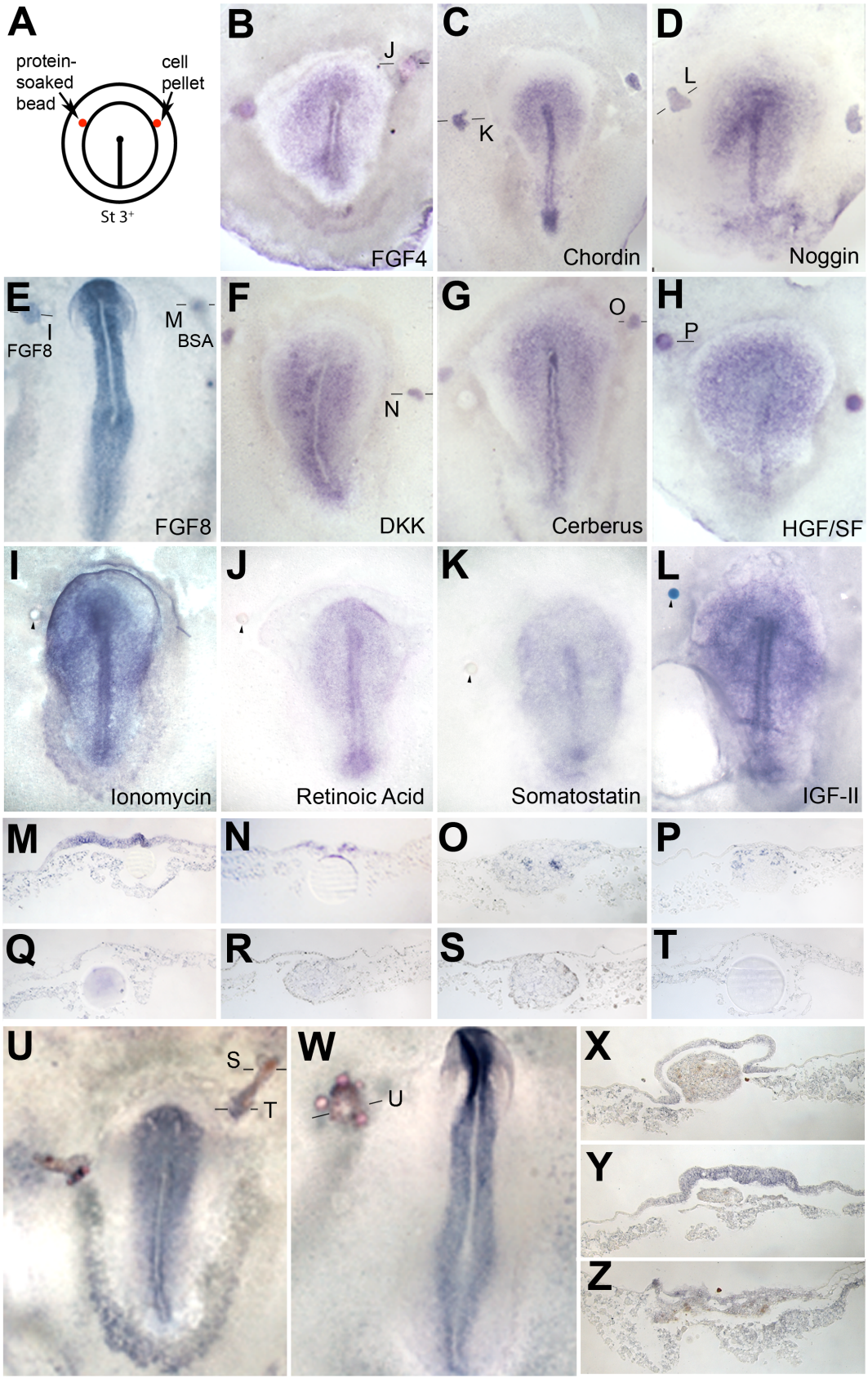

Fig. 3.10. Regulation of *Obelix* by various secreted factors.

Fig. 3.10. Regulation of *Obelix* by various secreted factors. A-P. The ability of various peptide factors to induce *Obelix* expression was tested by local application of beads soaked in the protein or pellets of COS-1 cells transfected with a construct encoding the factor into the area opaca of a host embryo (A). Examples of FGF4 beads (B), Chordin (C) and Noggin (D) cells, FGF8/control beads (E), Dickkopf (F), Cerberus (G) cells and HGF/SF bead. (H), ionomycin (I), retinoic acid (J), Somatostain (K), IGF-II (L) are shown. M-S show sections through the grafted region of the embryos in B-H at the levels indicated **U-Z**. Co-transplantation of a quail Hensen's node with beads soaked in the FGF inhibitor SU5402 has little or no effect: Obelix is still induced (U-Z). U shows a grafted embryo fixed after 6 hours, and W is an example of an embryo grown overnight after the graft. X-Z are sections through these embryos at the levels indicated in Q and R. Quail cells are stained with QCPN (brown). Note that some probes attach non-specifically to some types of beads and to COS cell pellets (eg. panels G-H).

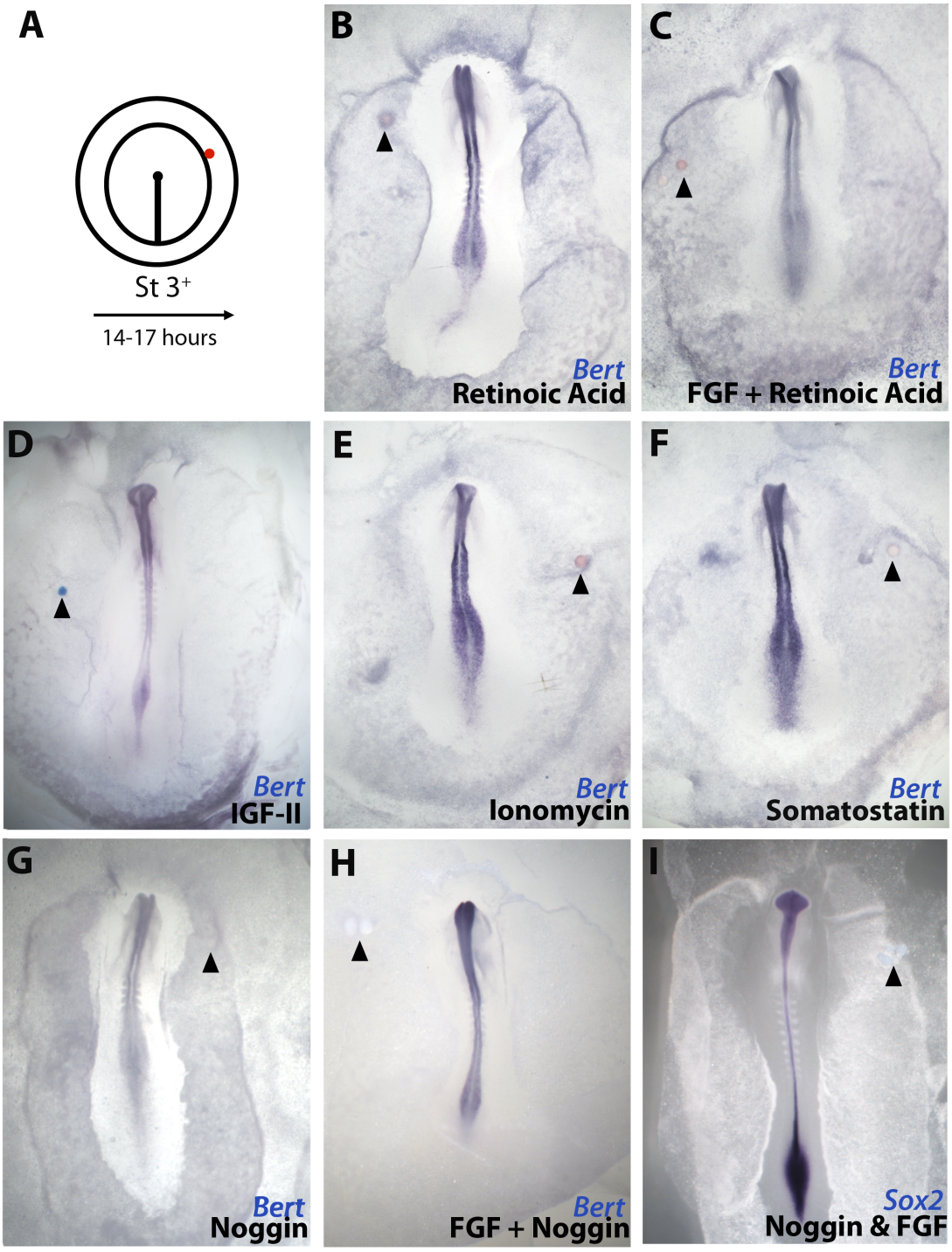

Fig. 3.11. Regulation of *BERT* by secreted factors.

Fig. 3.11. Regulation of *BERT* by secreted factors. The ability of various secreted factors to induce *BERT* was tested by local application of beads soaked in the protein into the area opaca of a stage 3^+ host chick embryo**. (**A) Diagram of experimental method. (B) *Bert* is not induced by retinoic acid. C, *Bert* is not induced by beads soaked in FGF and retinoic acid (arrow). (D) *Bert* is not induced by insulin growth factor-II (arrow), (E), *Bert* is not induced by ionomycin (arrow), (F), Bert is not induced by somatostatin (arrow), (G), *Bert* is not induced by a high concentration of the BMP inhibitor Noggin (arrow), (H) Bert is not induced by a combination of FGF and Noggin (arrow), (I), *Sox2* is not induced by beads soaked in high concentration of the BMP inhibitor Noggin in combination with FGF soaked beads (arrow).

Discussion

To understand neural induction it is important to identify both the nature and source of the signals that instruct cells to adopt a neural fate as well as the response of the cells that are induced towards neural fate. A differential screen was designed to identify the early responses to neural induction signals upstream of BMP inhibition (Streit et al., 2000). In total, 10 genes were shown to be up-regulated in response to the first 5 hours of signalling by a grafted node. To date nine of these have been characterized: *ERNI, Churchill, Calfacilitin, Dad1, polyubiquitin, ferritin heavy chain*, *Obelix, Asterix* and *TrkC* comprising the final one to be analysed (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010) Papanayotou, unpublished observations). Importantly, all the genes considered to date are expressed in the nervous system at early stages of development, which verifies that the screen has indeed identified genes expressed during normal neural development and are not artefacts of the experimental manipulation. Similarly, *TrkC* is in the prospective and early neural plate during development, and its expression can be induced in response to a grafted node in the area opaca epiblast.

Now that all 10 early response genes recognized by the screen have been characterized, some observations can be made on the early events of neural induction. First, it is clear that there is a temporal hierarchy of responses to the node. Indeed, the early response genes can be clustered into two classes on the basis of their expression during normal development. Some of the genes (*ERNI, Sox3* and *Calfacilitin*) are expressed very early in development, prior to the formation of the primitive streak ("pre-streak" group in Fig. 3.12), whilst a cohort genes are not expressed until mid-late primitive streak stages. *TrkC* and *Obelix* fall in to this later group (the "streak" group in Fig. 12) (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010). *Asterix* should also be included in this second group as although it is expressed early, this expression is only in an extraembryonic tissue (the hypoblast), and its expression in prospective neural tissue is similar to the other streak group members at stage 3 - 3^{+}.

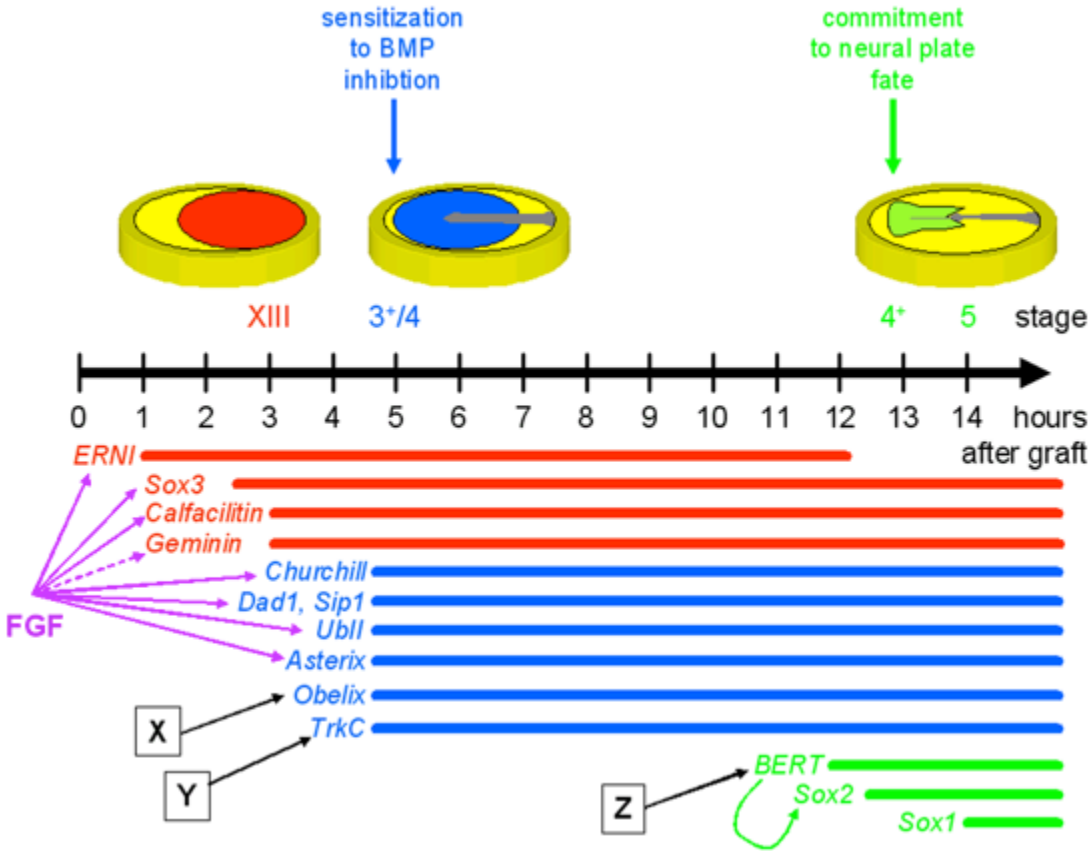

Fig. 3.12. Time-course of markers during neural induction and their regulation by signals. Temporal hierarchy of deployment of 13 early neural markers. The coloured lines at the bottom of the figure represent the period of expression of these genes, in relation to the time at which they are induced following a graft of Hensen's node into the area opaca (in hours on the scale above) and in relation to the stage of normal embryos at which they are expressed (stage shown above the time line). The diagrams above these stages schematize the domains of expression. The genes fall into three "epochs": those induced by a node within 3 hours start to be expressed in normal embryos before streak formation (red). Those induced by a node in 4-5 hours begin their expression at the mid- to late-primitive streak stage (blue) and those that are induced by a node at 12-13 hours do not begin their expression until the end of gastrulation, in the forming neural plate (green).

Interestingly, the temporal hierarchy observed in the expression of these two sets of genes, early "pre-streak" and later "streak" sets, is mirrored in their

induction by a grafted organizer (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010). The “pre-streak” group genes are induced within 3 hours (*ERNI* in 1-2 hours, *Sox3* and *Calfacilitin* in 2-3 h hours) (Streit et al., 2000; Papanayotou et al., 2010), while the remaining genes require 4-5 hours of exposure before they are robustly induced by a grafted node (Sheng et al., 2003; Gibson et al., 2010). Thus, it is clear that *Asterix*, *Obelix* and *TrkC* all fit into the second group as they are only weakly, if at all, induced by 3 hours, but robustly expressed 4-5 hours after grafting. Thus, it can be seen that the early response screen to neural induction has revealed two phases or “epochs” of gene expression during neural fate acquisition. A final epoch or phase could be considered to be the expression of *BERT* and *Sox2*, both of which are expressed later in development (stage 4^{+}- 5) and require at least 12 hours of exposure to signals from a grafted node (Papanayotou et al., 2008).

Neural induction has often been considered as a single step, the conversion of ectoderm to neural tissue mainly due to organizer grafting experiments in amphibians (Hemmati-Brivanlou and Melton, 1997a; Harland, 2000). Furthermore, BMP inhibitors have been proposed to mediate this conversion both experimentally and during normal development (Hemmati-Brivanlou and Melton, 1997a; Harland, 2000). However in the chick, the extraembryonic area opaca epiblast can be converted to a neural fate by signals from a grafted node (Waddington, 1930; Waddington, 1933). BMP inhibitors are unable to have any effect on directing this tissue to a neural fate unless they have already received 5 hours of signals from a grafted node (Streit et al., 1998). The differential screen has identified a cascade of early responses to neural induction signals upstream of BMP inhibition, of which seven of the previously identified genes have been shown to be induced by a source of FGF8 signals. Furthermore blocking FGF prevents their induction by a grafted node, suggesting that an FGF signal is both sufficient and required for their induction (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010) Panayotou et al., unpublished). This is also the case for *Asterix*, strengthening the conclusion that FGF is a key early signal in neural induction.

Together, these results argue that many of the initial effects of the node are due to FGF. Indeed, FGF8 is expressed at the right time during development in the node to be responsible for its inductive effects. However, the initial epoch of pre-streak genes is expressed prior to the formation of the node, thus the node itself cannot be the source of the initial signals during normal development. Another tissue, the hypoblast, is probably the source of FGF8 in the early embryo; indeed grafts of the hypoblast can induce both *Sox3* and *ERNI* but not later neural plate markers (Streit et al., 2000; Albazerchi and Stern, 2007), which makes it likely that during normal development the hypoblast initiates neural induction by inducing the first genes in the neural induction cascade through FGF signalling (Knezevic et al., 1995; Knezevic and Mackem, 2001; Stern, 2006; Albazerchi and Stern, 2007) Furthermore this fits with other studies that have suggested that FGF is required for neural induction both in chick (Streit et al., 2000; Wilson et al., 2000) and *Xenopus*

(Launay et al., 1996; Sasai et al., 1996; Linker and Stern, 2004; Delaune et al., 2005).

The role of BMP inhibition in the neural induction cascade is unclear as unlike FGFs, no combination of BMP antagonists is able to induce any neural markers in the chick area opaca (Streit et al., 1998; Streit and Stern, 1999; Linker and Stern, 2004). However, after 5 hours of exposure to signals from the node or a source of FGF, they can extend the expression of transiently induced genes (Streit et al., 1998). Given that no known markers are expressed in response to BMP antagonists the mechanism controlling this effect of BMP inhibition is unclear. If it provides just a permissive role then the question of why a minimum of 5 hours of signals from the node is required still needs to be elucidated. However, it is clear from experiments in chick (Streit et al., 1998; Streit and Stern, 1999) and *Xenopus* (Hawley et al., 1995; Sasai et al., 1995; Launay et al., 1996; Khokha et al., 2005; Reversade et al., 2005) that BMP inhibitors are a key part of the signals required for neural induction, and that they are required downstream of FGF in the neural induction cascade (Streit et al., 1998; Linker and Stern, 2004; Delaune et al., 2005).

However, it is only the signals from a grafted organizer (Waddington, 1930), that are sufficient to induce a stable, as well as fully patterned, secondary nervous system in the area opaca epiblast. Neither exposure to the hypoblast layer (Albazerchi and Stern, 2007), or to a source of FGF together with BMP inhibitors (Linker and Stern, 2004) are sufficient to induce a stable as well as fully patterned secondary nervous system in the area opaca epiblast. Thus, additional signals in addition to FGF and BMP inhibitors must be responsible for neural induction by a grafted node. Indeed, *Obelix* is only weakly induced by FGF, but its induction by a node cannot be blocked by a chemical inhibitor of FGF receptors, SU5402, suggesting that *Obelix* may be induced by a different activator of the MAPK pathway, such PDGF, or Insulin-like growth factors (IGF) other than IGF-II, which have also been suggested to have neuralising ability (Pera et al., 2001; Pera et al., 2003). However, there are still two genes identified in the response screen that cannot be induced by a

source of FGF: *TrkC*, and *Bert*. Thus, these are now useful markers to help identify the missing signals in neural induction.

Conclusion

In conclusion, the response to neural induction is a complex cascade of genes that are expressed with a temporal hierarchy reflected in both their expression during normal development in prospective neural tissue and in the timing of their induction by a grafted node. The response genes can be grouped into at least three phases or epochs of expression. FGF is a key neural inducing signal as it is sufficient and required to induce many of the response genes; however several of the response genes are not regulated by FGF. BMP inhibition has a role downstream of FGF but no neural markers are expressed in response to it. Thus, several key questions remain including what are the missing signals that regulate the remaining genes in the neural induction cascade? What mediates the response of cells to BMP levels? And to what cell states the various epochs of prospective neural plate development correspond?

Chapter Four

The role of Smad protein interactions in neural cell fate decisions

Introduction

In *Xenopus* a model whereby BMP antagonists secreted from the organizer direct nearby cells in the ectoderm to a neural fate has been proposed (Hemmati-Brivanlou and Melton, 1997a). Evidence for this includes the observation that cells in the animal cap ectoderm will express neural markers when exposed to BMP antagonists (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994). However in the chick, BMP antagonists are unable to induce neural markers in the area opaca epiblast, a region competent to respond to neural inducing signals from a grafted node (Streit et al., 1998; Streit and Stern, 1999; Linker and Stern, 2004). Area opaca cells are only able to respond to BMP antagonists if they have first been exposed to a minimum of 5 hours of signals from the node (Streit et al., 1998), after which BMP antagonists are able to extend the expression of neural markers transiently induced by the node.

To characterize the molecular basis of this sensitization to BMP signalling, a differential screen was designed, comparing area opaca epiblast that had been exposed to 5 hours of signals from a grafted node to the contralateral area opaca epiblast that had not received such signals (Streit et al., 2000). Of the 10 genes identified in this differential screen, the best candidate to explain the change in the ability of cells to sense the level of BMP signalling is *Churchill*, acting through SIP1, Smad-interacting protein 1(Sheng et al., 2003).

Sip1 is a zinc-finger homeodomain protein expressed in the prospective neural plate and up-regulated by the transcriptional activator *Churchill* after 4-5 hours of signalling from the node (Sheng et al., 2003). Sip1 has been

reported to act as a transcriptional repressor of mesodermal genes (Verschueren et al., 1999; Sheng et al., 2003), and was suggested to protect nascent neural tissue from a mesodermal fate, thereby forming the boundary at the mid-line of the embryo between neural and mesodermal tissue (Sheng et al., 2003). Sip1 was originally identified in a screen for binding partners of phospho-SMAD1 (Verschueren et al., 1999) - the activated form of the intracellular protein SMAD1, downstream of the BMP receptor. Furthermore, it has been reported that Sip1 can inhibit BMP signalling through an interaction with SMAD1 in cell culture assays (Postigo, 2003; Postigo et al., 2003). Given that its is induced by a node (Sheng et al., 2003) at a time that corresponds to when cells become responsive to BMP signalling (Streit et al., 1998), could Sip1 explain the difference between cells that have or have not received 5 hours of signalling from the node?

The level of BMP signalling, and therefore of phosphorylated SMAD1, is low in the neural plate, but high at its lateral boundary with epidermal tissue where neural crest and sensory placodes arise (Streit and Stern, 1999; Faure et al., 2002). Could Sip1 be responsible for forming the boundary at the lateral edge of the neural plate between neural and epidermal fates?

Neural tissue can be seen as having two boundaries; the lateral edge of the neural plate with the epidermis, and the midline of the embryo where it abuts the mesoderm. At the lateral border BMP (and therefore Smad1) levels are particularly high, whereas close to the primitive streak prospective mesodermal cells are exposed to Nodal/Activin, which act through Smad2 (Stern et al., 1995). It has been proposed that Smad1 and Smad2 compete for binding to the common mediator Smad4 (Candia et al., 1997), which is required for transduction of both BMP and Nodal signals, and that an increase in one signalling pathway can cause a decrease in the other. Could the cell fate decisions between mesodermal, neural and epidermal fates, which define the midline and lateral boundaries of the neural plate, be controlled by the balance between relative levels of activated Smad1 and Smad2 through a see-saw like mechanism, involving competition for Smad4?

Design of Experiments

The foregoing paragraphs present two questions relating to the possible roles of Smads and Sip1 in regulating neural fate and its borders during neural induction. The experiments in this chapter were designed to address these questions, as follows:

Is Sip1 responsible for the ability of cells to sense BMP levels through an interaction with phospho-SMAD1?

To investigate whether Sip1 could act as a sensor for BMP signalling, the full-length coding sequences of SIP1 and Smad1 need to be cloned into vectors containing complementary fragments of the Venus protein for a bi-molecular complementation (BiFCo) interaction assay (Hu et al., 2006), with truncated versions of the *Smad* sequence lacking interaction domains as a negative control. A positive interaction between these constructs when transfected into cells would reveal this interaction in living cells, and allow the effect of varying the level of BMP to be investigated. Furthermore, electroporation of constructs into chick embryos would enable the localization and dynamics of interaction events at the neural plate boundary to be observed in vivo. If successful, mutated Smad1 constructs that are impervious to phosphorylation, or their constitutively active counterparts, could be created, as previously described (Pera et al., 2003), to assess whether the interaction involves differential binding of Smad1 according to its phosphorylation status .

Do Smad1 and Smad2 compete for binding to Smad4?

To determine whether Smad1 and Smad2 compete for binding to Smad4, plasmids containing the coding sequence of these genes cloned into vectors containing complementary fragments of the Venus protein would enable the BiFCo interactions between Smad1 and Smad4, and Smad2 and Smad4 constructs to be visualized by transfection into a cell line. The dynamics of a competitive interaction can then be investigated by stimulating the

phosphorylation of the competing Smad, by addition of BMP or Activin/Nodal ligands. If successful, multi-colour bi-molecular complementation analysis could be used to evaluate the dynamics of competitive Smad1-Smad4-Smad2 interaction.

Methods

Cloning of BiFCo constucts.

Cloning of pcDNA3.1XSip1venusC

Bi-molecular fluorescence complementation (BiFCo) assays (Hu et al., 2002) to assess protein-protein interactions in vivo require cloning to create proteins conjugated to either the N-(amino acids 1-154) or the C-(amino acids 155-half of the fluorescent protein Venus (a variant of yellow fluorescent protein, EYFP) (Saka et al., 2007). The full length, 3642 bp, open reading frame of *Xenopus* Smad1 interacting protein 1, SIP1 (NCBI accession number NM_001098675) (Verschueren et al., 1999; Sheng et al., 2003)) was sub-cloned into the pcDNA3.1venusC plasmid, in frame to the 275 bp venusC (VC155) sequence by PCR amplification and the engineered cut sites NheI and EcoRV.

Primers were designed (Invitrogen) to amplify the XSIP1 coding sequence lacking the stop site using Accuprime Pfx DNA Polymerase (Invitrogen).

The primer pair:

Forward: TACGCTAGCCATGAAGCAAGAGATCATGGCGGATGGCTAC.

Reverse: TTTTGATATCCATGCCATCCTCCATTATCTC.

were used in a reaction conditions of 95°C for 2 min followed by 27 cycles of [95°C 15 sec, 64°C 30 sec, 68°C 4 min]. The band amplified (about 4 kb) was gel purified and ligated into the pcDNA3.1venucC vector that had been opened with NheI and EcoRV digestion, using rapid ligation mix (Promega) for 15 min at room temperature, and transformed mix into Top10F' competent cells. Colonies containing the pcDNA3.1XSIP1venusC plasmid were selected

by ampicillin resistance. Finally the sequence of the plasmid was sequenced using 8 pairs (forward and reverse orientations) of primers to ensure that the full length XSIP1 sequence was in frame with venusC.

Cloning of pcDNA3.1cSmad1venusN

The 1398 bp chick *Smad1* open reading frame (NCBI Accession Number: AY953143) was extracted from a chick DNA library by PCR using a primer set designed to amplify Smad1 excluding its 3' stop site. A designed (Invitrogen) primer set:
Forward: ACTCAGTCTAGAGCGGCCGCATGAACGTGACAAGTTTATTTTCCTTCACC,
Reverse: ACTCAGGATATCCCAGACACTGAAGAAATAGGATTATGAGG, amplified a single product of approximately 1.5 kb using *Taq* polymerase (30 cycles of [30 sec 94°C, 30 sec 42°C, 5 sec 72°C]). The PCR product was then purified by gel extraction and cloned into pGem-Teasy (Invitrogen). The plasmid was transfected into XL10-gold competent bacteria (Invitrogen), and positive colonies were selected by blue/white selection on ampicillin plates. The cSmad1 sequence was cut from the pGem-Teasy vector using the engineered restriction sites XbaI and EcoRV and ligated into pcDNA3.1venusN to create the pcDNA3.1.cSmad1venusN plasmid.

Cloning of pcDNA3.1cSmad1δMH2venusC

The MH2 domain of chick Smad1 (NCBI Accession Number AY953143) was identified by sequence homology to the well characterized MH2 domain of Drosophila and *Xenopus* proteins (Meersseman et al., 1997) and primers were designed (Invitrogen) to amplify only the 792 bp sequence encoding cSmad1 amino acids 1 – 264, lacking the MH2 domain. The Smad12δMH2 sequence was amplified from a chick stage 2-4 DNA phage library by PCR. The designed forward [ACTCAGTCTAGAGCGGCCGCATGAACGTGACAAGTTTATTTTCCTTCACC] and reverse [ACTCAGGATATCCCAGCAACAGCCTGAACATCCTC] primers amplified a single product of about 800 bp using *P. fu* polymerase

(Invitrogen) [1 min 94°C, followed by 31 cycles of 1 min 94°C, 1 min 46°C, 8 min 72°C]. The PCR product was purified by gel extraction and cloned into the pCR-blunt (Invitrogen) plasmid by overnight incubation (5 μl ligation buffer, 3 μl PCR product, 1 μl pCRblunt plasmid, 1 μl T4 DNA ligase), then transformed into XL-10 Gold competent bacteria (Invitrogen) and selected with blue/white selection on kanamycin plates. Finally, the Smad1δMH2 sequence was cut from the pCRblunt plasmid through the engineered cut sites XbaI and EcoRV and ligated into the pcDNA3.1venusN to create pcDNA3.1cSmad1δMH2venusN.

BiFCo plasmids

Additional plasmids used in the BiFCo interaction experiments were kind gifts of Professor Jim Smith: pCS2+VenusN-humanSmad4, pCS2+VenusC-*Xenopus*Smad2, pCS2+VenusC155-*Xenopus*Smad3 (Saka et al., 2007) pCS2+VenusN-Smad2δSXS, pCS2+VenusC-Smad4 (Harvey and Smith, 2009).

COS cell culture and transfection for BiFCo experiments

Cell culture and transfection of plasmids for Bi-molecular fluorescence complementation assays were carried out as previously described (Streit et al., 1998; Papanayotou et al., 2008), using the COS-1 Competent Origin SV40), stable, immortal mammalian cell line originally derived from Green monkey *Chlorocebus sabaeus* epithelial kidney cell line (Gluzman, 1981). COS-1 cells were maintained in DMEM medium (Dulbecco's Modified Eagle's Medium: Gibco) containing 10% Fetal Calf Serum (Pan) and 2 mM Glutamax (Invitrogen). Cells were grown to 50-60% confluence in 6-well tissue culture plates then passaged and plated at a density of 1.5 x 10^5 cells/ well containing glass coverslips. Cells were allowed to grow overnight before being transfected with the BiFCo constructs using Opti-MEM (Invitrogen) and lipofectamine (Invitrogen). For a single interaction pair of plasmids, 1.5 μl of

each plasmid were each mixed with 100 μl of Opti-MEM solution and 6 μl of PLUS reagent (Invtrogen) before being allowed to settle at room temperature for 15 min. After 15 min the contents of both Eppendorf tubes were combined with 4 μl of Lipofectamine and the mixture was removed from the cells and fresh 800 μl medium added, then 200 μl of the plasmid-lipofectamine solution was added to the COS cells growing on the coverslips. The cells were incubated in this solution for 4 hours at 37°C then the medium replaced with 2 ml of DMEM (10% FCS, 2 mM Glutamax) including any additional medium supplements where indicated. Bert-venusC, ERNI-venusN constructs were used as positive controls (Papanayotou et al., 2008). Epifluorescence was visualized under a compound fluorescence microscope or by confocal microscopy in live cells by inverting and mounting coverslips on a standard slide either 4 hours post- transfection or after overnight incubation.

Results

Is Sip1 responsible for the ability of cells to sense BMP levels through an interaction with phospho-Smad1?

To investigate the interaction of Sip1 and Smad1 proteins Bi-molecular Fluorescence Complementation (BiFCo) analysis might be provide a useful methodology. BiFCo assays rely on creating chimaeric protein complexes with fragments of the fluorescent protein, Venus (Hu et al., 2002; Kerppola, 2006). The fragments by themselves are not fluorescent, however if the two tagged proteins interact they bring the two halves of Venus protein into proximity and fluorescence is restored, enabling the visualization of protein-protein interactions in living cells, in cell culture (Papanayotou et al., 2008), or in vivo, as has been done in *Xenopus* (Saka et al., 2007) and zebrafish (Harvey and Smith, 2009) embryos. First, the sequences of both proteins needed to be cloned into constructs to create fusion proteins that can be used in the protein-protein interaction assay.

The *Xenopus* SIP1 sequence varies from the published sequence.

Full length *Xenopus* SIP1 has previously been isolated and shown to act functionally in the chick (Sheng et al., 2003). Therefore, for BiFCo interaction assays, it was subcloned, removing its transcriptional stop site and fused in frame with the C-terminus of Venus. To confirm that the cloned sequence was indeed in frame, the construct was sequenced by 8 pairs of overlapping forward and reverse primer sequences. Whilst it was confirmed that the sequence had been cloned in frame, it was also noted that the sequence varied from the published full length *Xenopus* sequence (NM_001098675) the translation of which would result in a total of five amino acid changes Amino acids 131-134 TRSV (NM_001098675) were NNGT, and single amino acid change was seen at residue 168 Q (NM_001098675) was R. However, when this variant amino acid sequence was compared to both human, mouse and chick homologues of SIP1 (also known as *Zeb2*, or *Zfhx1*) all five of the variant amino acids 131-134 and 169, were found to be present in these sequences (Fig. 4.1). Thus, the original clone was completely re-sequenced to confirm the sequence variants in the original plasmid. Thus, it is possible that the *Xenopus* SIP1 sequence is more similar to mouse and human homologues than had previously been thought. Nonetheless the amino acids variant are not in the region that has previously been associated with SIP1s ability to bind SMADs (Smad-binding domain, SBD) (Verschueren et al., 1999), a region that shares a high degree of homology with human, mouse and chick sequences (Fig. 4.2). Given the identity to the mouse and human sequence at the variant amino acid residues it was decided that this construct was still suitable for interaction studies.

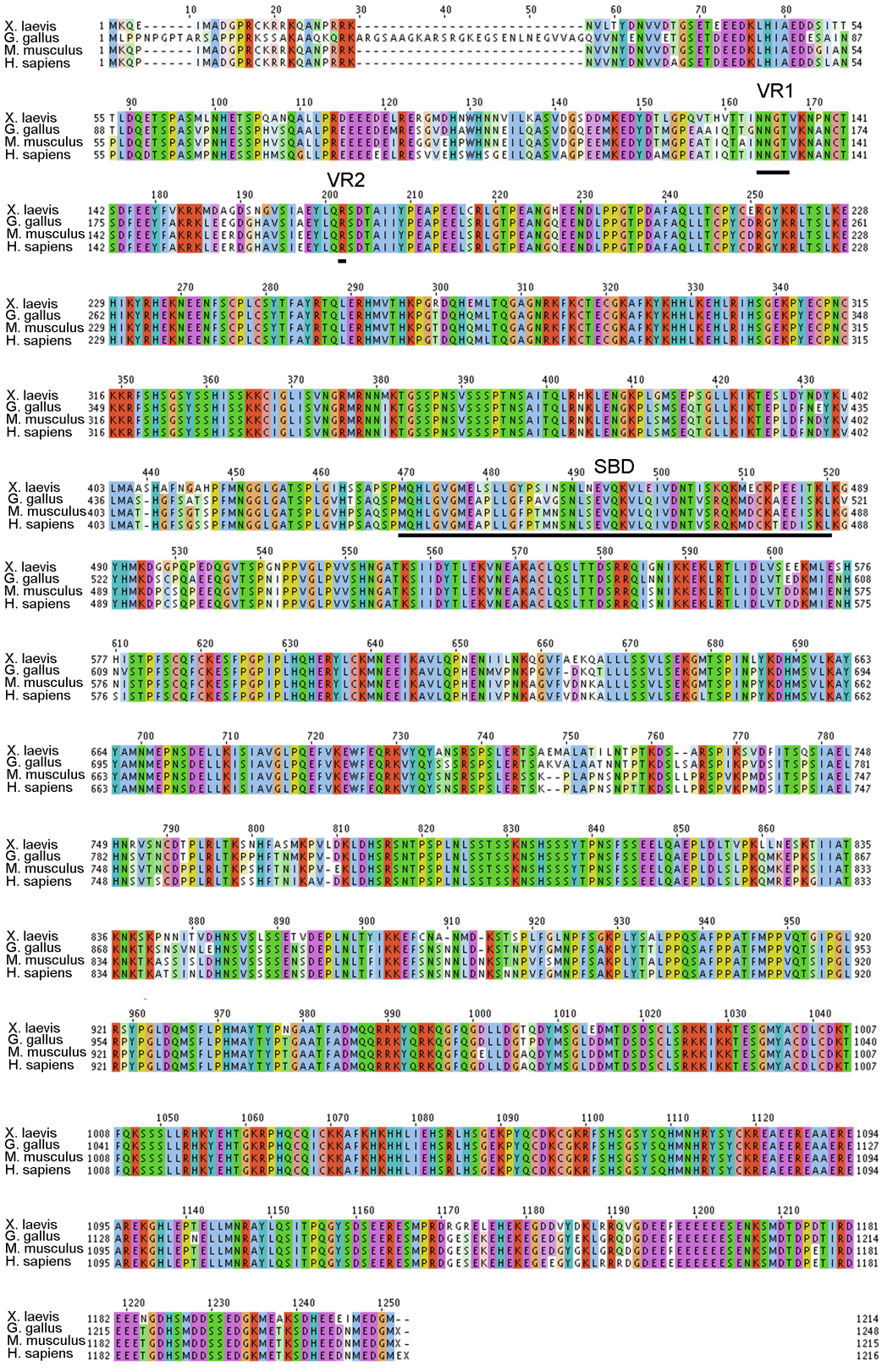

Fig. 4.1. SIP1 Amino Acid Species Comparison

Figure 4.1. SIP1 Amino Acid Species Comparison. An amino acid alignment of *Xenopus laevis* SIP1 from nucleotide sequencing data including the two variant regions (VR1, VR2), aligned with *G. gallus* ZFHX1B (XM_422151) *M. musculus* ZEB2 (NM_015753) and *H. Sapiens* ZEB2 (NM_014795). The previously identified Smad-binding domain has also been highlighted (SBD). Residues are displayed in different colours based on amino acid identity

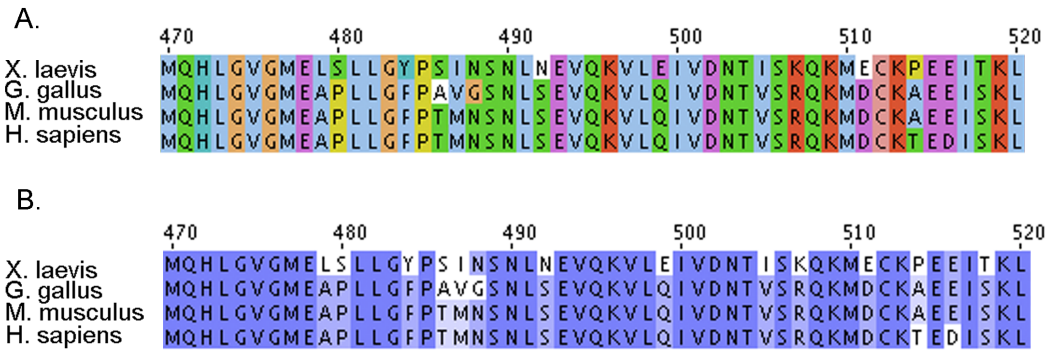

Fig. 4.2. Comparison of Smad-Binding Domains of SIP1 Homologues. Alignment of the Smad-Binding Domains (Postigo, 2003; Postigo et al., 2003) of *Xenopus laevis* (NM_001098675), *Gallus gallus* (XM_422151), *M. musculus* (NM_015753), and *H. sapiens* (NM_014795). (A) Residues are displayed in different colours depending on amino acid identity. (B) Residues are displayed in shades of blue depending on residue homology: (37/51) 72% identity across all four species.

Does SIP1 interact with Smad1 in vitro?

Sip1 is up-regulated after 5 hours of induction by a grafted node, the time in which cells can respond to the level of BMP. Furthermore SIP1 has been isolated in screens for binding partners of Smad1 (Verschueren et al., 1999) both of which can be co-immunoprecipitated in cell culture extracts (Postigo, 2003; Postigo et al., 2003). It was hypothesized that imaging this interaction by bi-molecular complementation assay (Hu et al., 2002; Kerppola, 2006) may reveal the site and the dynamics of any interaction between these two proteins in vivo, during the stages of neural fate acquisition. Moreover it would provide a tool to determine whether the interaction itself is sensitive to the presence of BMP.

To test the viability of BiFCo analysis as a tool for visualizing protein-protein interactions, plasmids containing the BERT-VenusC and ERNI-VenusN fusion proteins that have previously been shown to interact (Papanayotou et al., 2008), were tested in vitro by transfection into a stable COS cell line and observed for fluorescence 24 hours post transfection. As previously reported (Papanayotou et al., 2008), cells transfected with these constructs show fluorescence (Fig 4.3. A), whilst empty vectors containing only the halves of the Venus protein do not (Fig 4.3. B), suggesting that fluorescence seen in cells transfected with ERNI and BERT does require binding of the tagged proteins.

To investigate if an interaction between SIP1 and Smad1 could be visualized by BiFCO assay in the absence of additional stimulation of BMP signalling, COS cells were transfected with a combination of SIP1VenusC and Smad1VenusN plasmids and cultured in medium without additional BMP. As controls, a mutated form of Smad1 lacking its MH2 protein-interaction domain (Smad1δMH2venusN) and Smad2venusN, neither of which should interact with SIP1, were used as negative controls for non-specific binding, BERT and ERNI constructs were used as positive controls for the transfection procedure. After transfection, cells were observed for fluorescence at 4, 24 and 48 hours. Despite fluorescence in BERT and ERNI positive controls, no fluorescence

was seen at any time point in SIP1VenusC-Smad1VenusN transfected cells (Fig 4.3. C), or in negative controls (Fig 4.3. C, D) suggesting that under these conditions SIP1 and SMAD1 do not interact.

Does Smad1 need to be phosphorylated to interact with SIP1?

It has been suggested that the phosphorylation of Smad1 is required to enable an interaction with SIP1 (Verschueren et al., 1999; Postigo, 2003; Postigo et al., 2003). BMPs binding to type-I transmembrane serine-threonine kinases cause the phosphorylation of Smad1. Therefore to test if BMP signalling can promote an interaction between SIP1 and Smad1 in the in vitro assay, COS cells co-transfected with Smad1VenusC and Sip1VenusN constructs were cultured in the presence of a low (50 ng/ml) or high (100 mg/ml) concentration of BMP4 for 4, 24, and 48 hours. BERT and ERNI plasmids were used as a positive control for the transfection procedure, Despite fluorescence in positive controls (Fig 4.4.D) no fluorescence was observed in transfected cells at either concentration of BMP4 (Fig. 4.4. A-C) at any of the time-points tested.

Its possible that BMP4 stimulation of Smad1 phosphorylation only acts transiently, therefore, cells were also transfected with SIP1 and Smad1 interaction constructs and cultured for 4 hours in un-supplemented medium; Smad1-phosphorylation was then stimulated by addition of BMP4 and observed for fluorescence after 15, 30 and 60 minutes. Again, no fluorescence was observed at any time-point (not-shown), suggesting that even in the presence of active BMP signalling, and presumably phosphorylation of the Smad1 proteins, no transient interaction with SIP1 occurs. Thus, these in vitro BiFCo assays were unable to uncover interactions between SIP1 and Smad1 proteins.

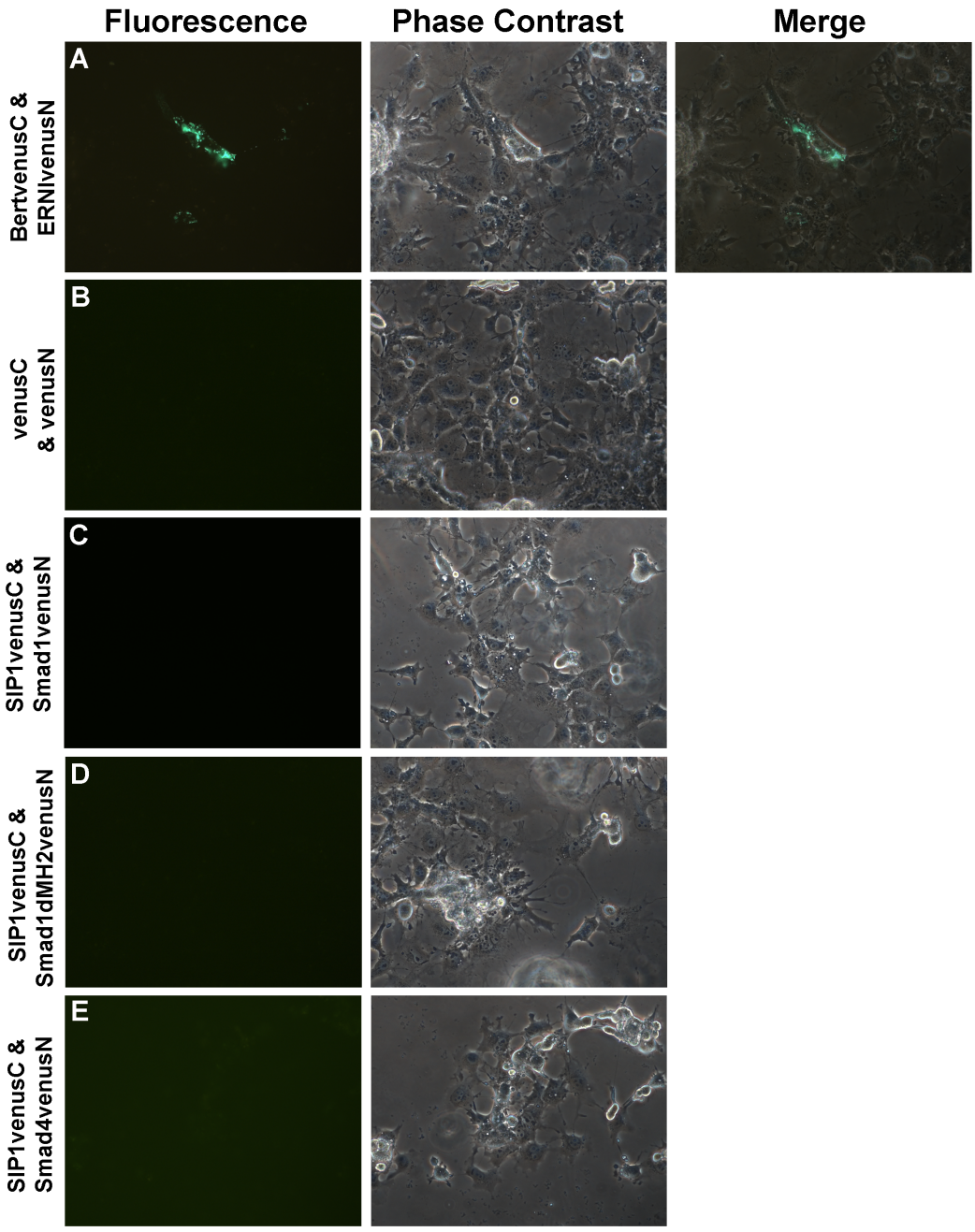

Fig. 4.3. SIP1 and Smad1 constructs do not interact in BiFCo interaction assays in unsupplemented medium conditions. COS cells were co-transfected with pairs of constructs, and cultured in medium unsupplemented with additional cytokines. (A) a positive interaction was observed between BERTVenusC and ERNIVenusN plasmids 24 hours post transfections, (B) a negative interaction was observed for the empty vectors with Venus fragments. No interaction was observed between SIP1VenusN and Smad1VenusC (C), or the negative controls Smad1δMH2VenusC (D) or Smad4VenusC. All images are representative of cultures.

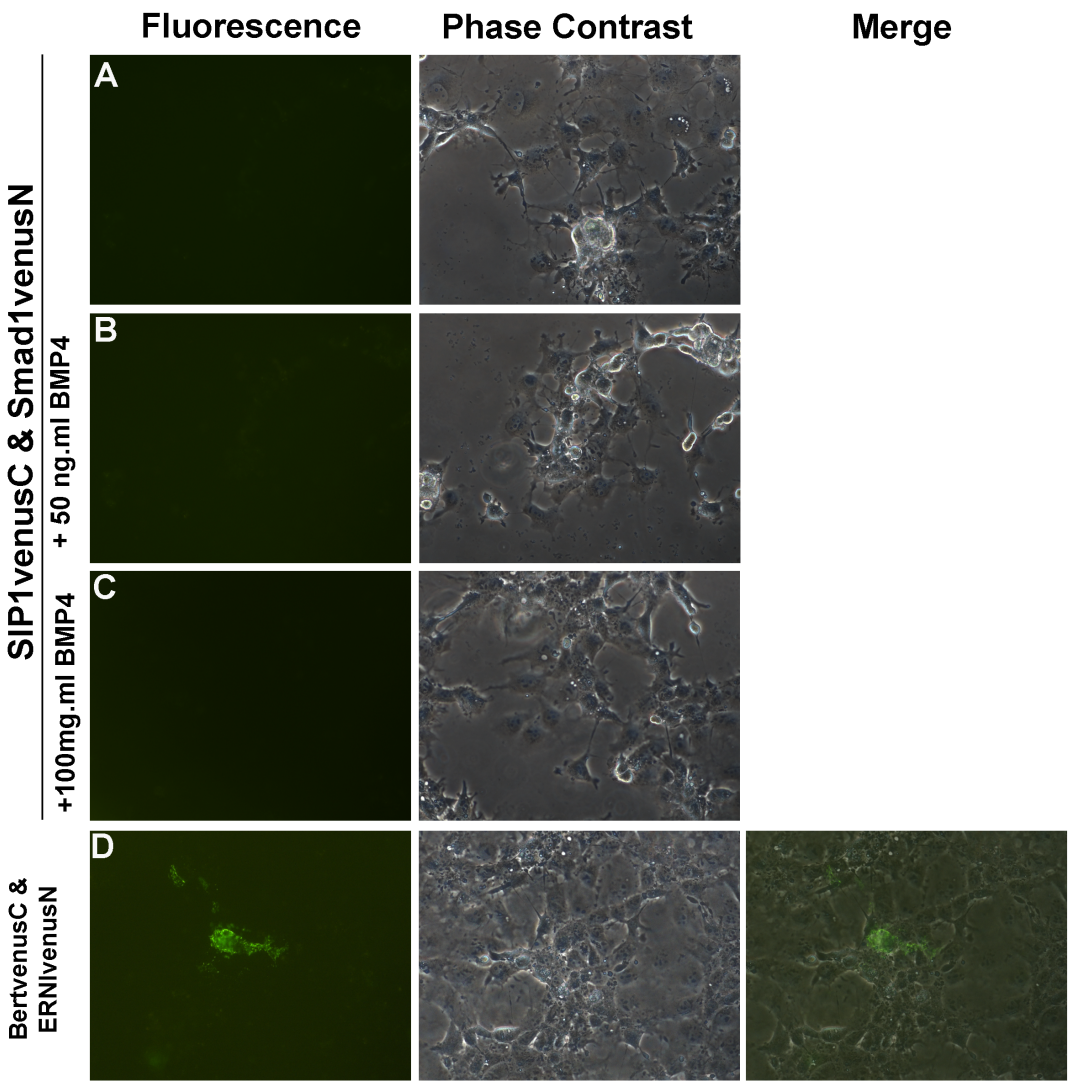

Fig. 4.4. SIP1-Smad1 interaction is not enhanced by stimulation of BMP4 in BIFCo in vitro assays. COS cells were co-transfected with pairs of constructs and observed for fluorescence over a time-course. No interaction was observed for SIP1VenusN and Smad1VenusC constructs when cultured in unsupplemented medium (A), or when cultured in the presence of (B) 50 ng/ml or (C) 100 mg/ml BMP4, although positive controls for the transfection procedure ERNIVenusC and BERTVenusN did interact (D). All images are representative of the cell populations and show cultures 24 hours post transfection.

Do Sip1-Smad1 interactions require a living cellular environment?

Previous studies have suggested that the activity of SIP1 as a binding partner of Smad1 is controlled by recruitment of co-factor proteins that control the specificity of its protein-protein interactions (Postigo et al., 2003; van Grunsven et al., 2007). Therefore it is possible that COS cells culture conditions do not provide the correct complement of co-factors to enable an interaction. Thus BiFCo studies were attempted in vivo in chick embryos to establish whether an interaction could be observed and if it would be localized to specific regions in the embryo. SIP1venusN and Smad1venusC constructs were electroporated into stage 3 chick embryos and observed for fluorescence at 5, 8, and 24 hours post-electroporation using dsRed as a marker for successful electroporation. However, even when constructs had been successfully electroporated (as shown by dsRed expression) no expression of Venus could be seen (0/21) (Figure 5.5). Fluorescence was also not seen if dsRed was not included (0/6), which was carried out in case of an inhibitory interaction. As a positive control Smad1venusN and Smad4venusC constructs, were electroporated in stage 3 chick embryos, however this combination did not produce observable levels of fluorescence (0/5). Thus, in summary, no observable interaction could be visualized with either experimental, SIP1-Smad1, or control plasmids Smad1-Smad4 in chick embryos.

Could the lack of interaction be specific to chick and/or mammalian cells?

Much of the previous work on SIP1-Smad1 interactions is based on work in *Xenopus* (van Grunsven et al., 2000; Papin et al., 2002; Nitta et al., 2004; van Grunsven et al., 2007). The failure of the above experiments to produce the expected interactions raises the possibility that these interactions are specific to anamnia and that they do not occur in mammalian (COS cells) or avian cells (chick embryos). Given that BiFCo interaction studies have been successfully carried out by microinjection in zebrafish embryos (Harvey and

Smith, 2009) and given that *D. rerio* Sip1 knockdown experiments result in neural crest defects (Delalande et al., 2008), zebrafish embryos were used for visualizing the SIP1-Smad interaction. 25 pg and 50 pg of SIP1venusN and Smad1venusC were co-injected into the animal pole of 1-cell stage zebrafish embryos. The embryos were then observed for fluorescence after 6, 8, and 24 hours. However, no fluorescence was observed in any injected embryos at either concentration or time-point (0/80). This was also the case for positive control Smad1venusN-Smad4venusC (0/20) and ERNI-BERT (0/20) plasmid co-injections. Thus, co-injection of BiFCo plasmids in zebrafish embryos did not provide a methodology for exploring the SIP1-Smad interaction in vivo, which further suggests that the lack of interaction observed in COS cells and chick embryos is not an amniote-specific problem.

Do Smad1 and Smad2 compete for binding to Smad4?

Smad1 and Smad2 transduce BMP and Nodal/activin signals, respectively, by forming complexes with the common mediator Smad, Smad4. Given that both pathways converge at Smad4 it has been speculated (Candia et al., 1997) that Smad1 and Smad2 competition for binding may provide cells with a mechanism for sensing the relative levels of these two signals. If so, the interaction of Smad1 with Smad4 should be inhibited by Smad2 and visa versa. Therefore to investigate whether a competitive interaction exists between Smad1 and Smad2 for Smad4 binding, and is affected by simulation of their opposing pathways, fusion constructs for BiFCo analysis were electroporated into a stable COS cell line to visualize the interaction in living cells and the level of TGFβ signalling was varied by addition of BMP or Activin.

To investigate if Smad1-Smad4 and Smad2-Smad4 interactions can be visualized in living cells, fusion constructs were co-electroporated into COS cells with and without the presence of BMP4 or Activin and visualized after 24 hours. As a negative control, mutated forms of Smad1 and Smad2 lacking

their protein-interaction domain, MH2 (Smad1δMH2 and Smad2δMH2), were each co-electroporated into COS cells along with Smad4.

Smad1 and Smad4 fusion constructs interacted when cultured in un-supplemented medium (Fig 4.6. A). In cells additionally treated with BMP4 no significantly effect was seen either on the level of fluorescence in positive cells or on the number of cells (approximately 50-75 cells per well) showing fluorescence (Fig. 4.6B). Similarly, the addition of Activin did not significantly affect the SMAD1-SMAD4 interaction as strong fluorescence was still seen in these cells (Fig. 4.6 C). However, the negative control Smad1δMH2 construct, also interacted with Smad4 in unsupplemented conditions (Fig. 4.6 D). Thus, the interaction between Smad1-Smad4 constructs was unaffected by treatment with either BMP4 or Activin; negative controls suggest that this may be due to non-specific binding.

Similar results were also obtained with Smad2-Smad4 interactions. Smad2 and Smad4 interacted in unsupplemented culture conditions (Fig. 4.7 A), but in the presence of Activin or BMP4 the Smad2-Smad4 interaction was not significantly altered (Fig. 4.7 C, D). Furthermore, some fluorescence was also observed in negative controls (Smad2δMH2-Smad4 (Fig. 4.7 D) although this was limited to only a few cells (2-3 per well). Therefore, similar to the Smad1-Smad4 interaction, the interaction of Smad2-Smad4 constructs, in these assays were not sensitive to stimulation by the addition of BMP4 and Activin.

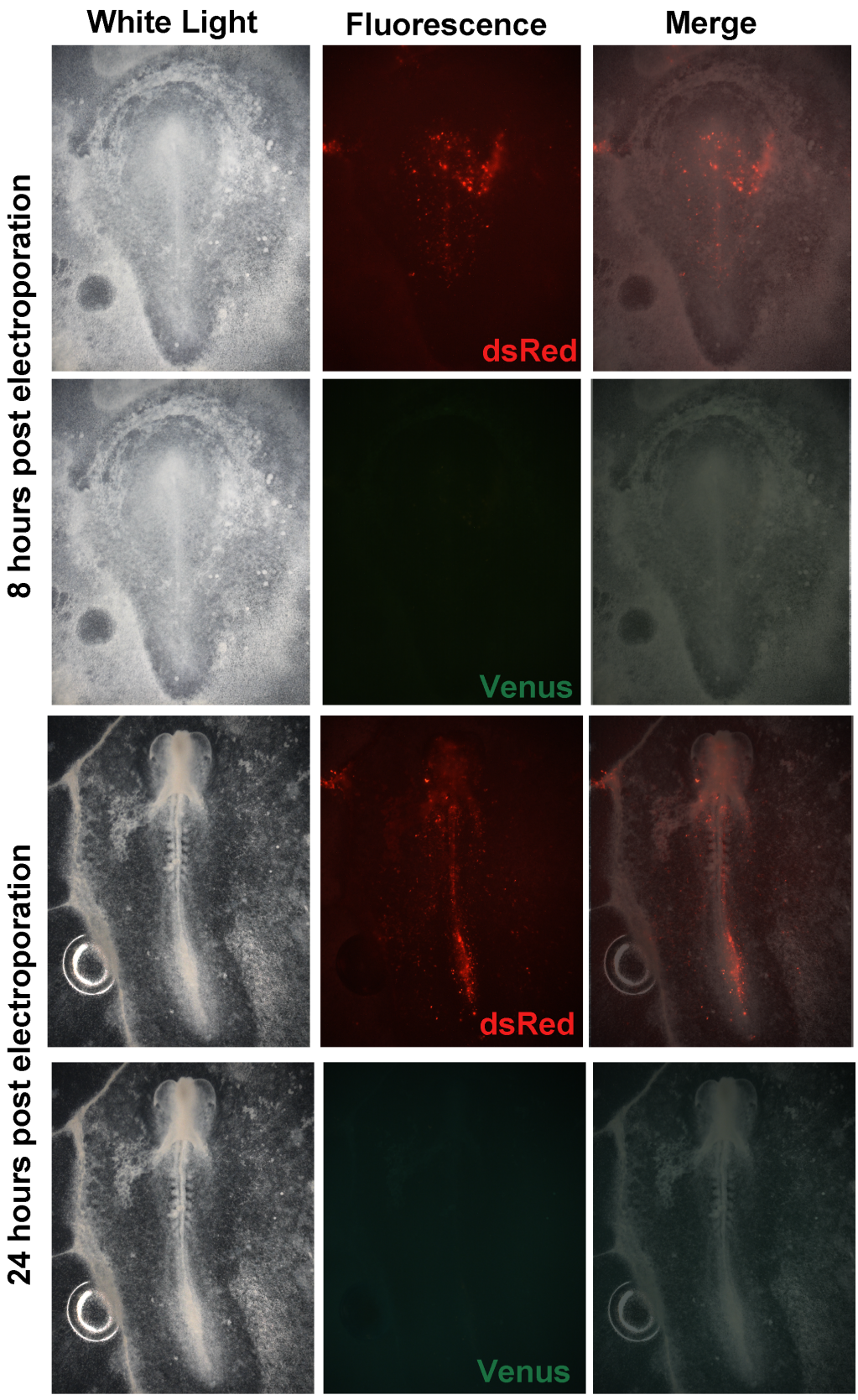

Fig. 4.5. In vivo BiFCo assay in chick embryos

Fig. 4.5. In vivo BiFCo assay in chick embryos. Stage 3 chick embryos were co-electroporated with dsRed as a marker for successful electroporations, together with plasmids containing SIP1VenusC and Smad1VenusN constructs. No fluorescence of the Venus plasmid was observed at 8 or 24 hours post-electroporation.

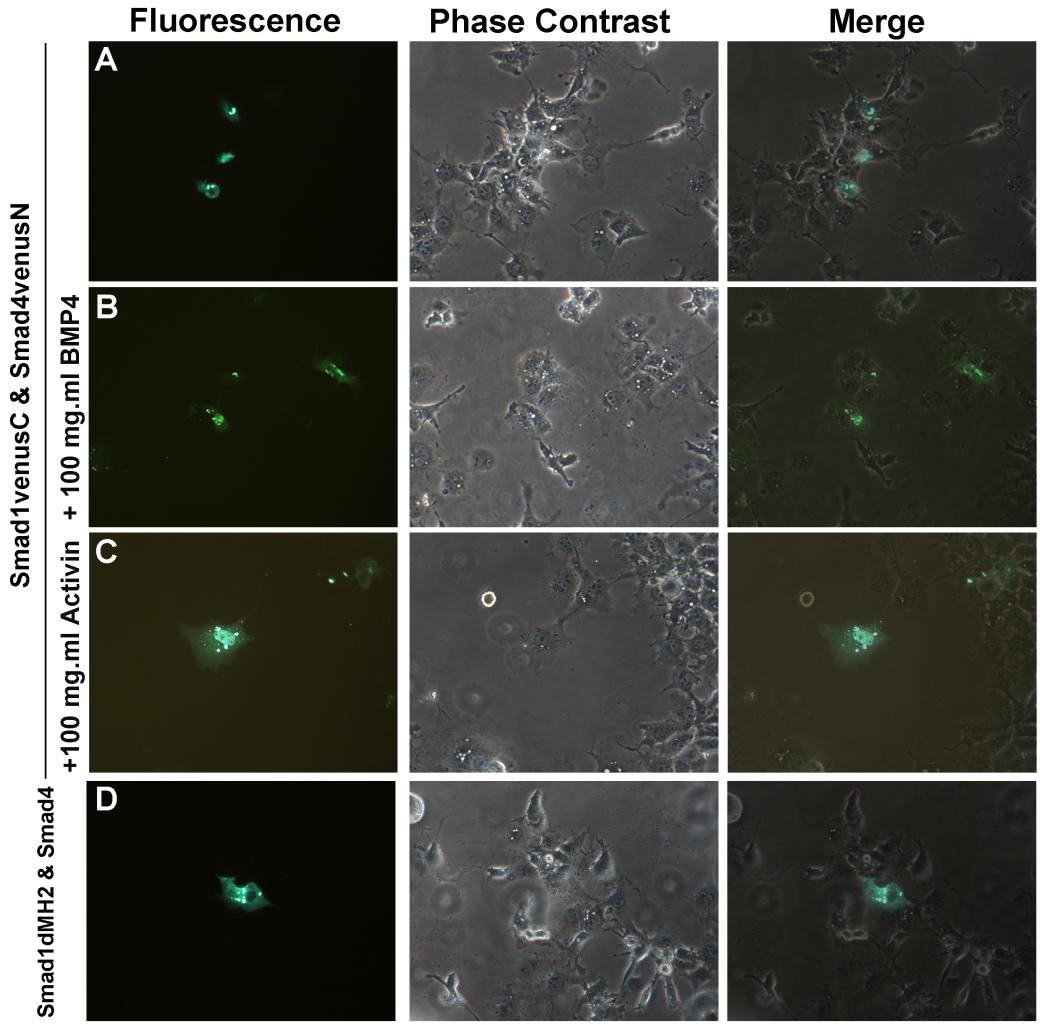

Fig. 4.6. Smad1-Smad4 BiFCo in vitro assays. COS cells were co-transfected with pairs of interaction constructs cultured in medium supplemented with BMP4 or Activin. Fluorescence was observed in cells without additional supplementation (A) and similarly when cultured with BMP4 (B). Fluorescence was still present, although in only a few cells when Activin was added to cultured cells transfected with Smad1 and Smad4 constructs. An interaction was also observed between the negative control construct Smad1δMH2 and Smad4 (D).

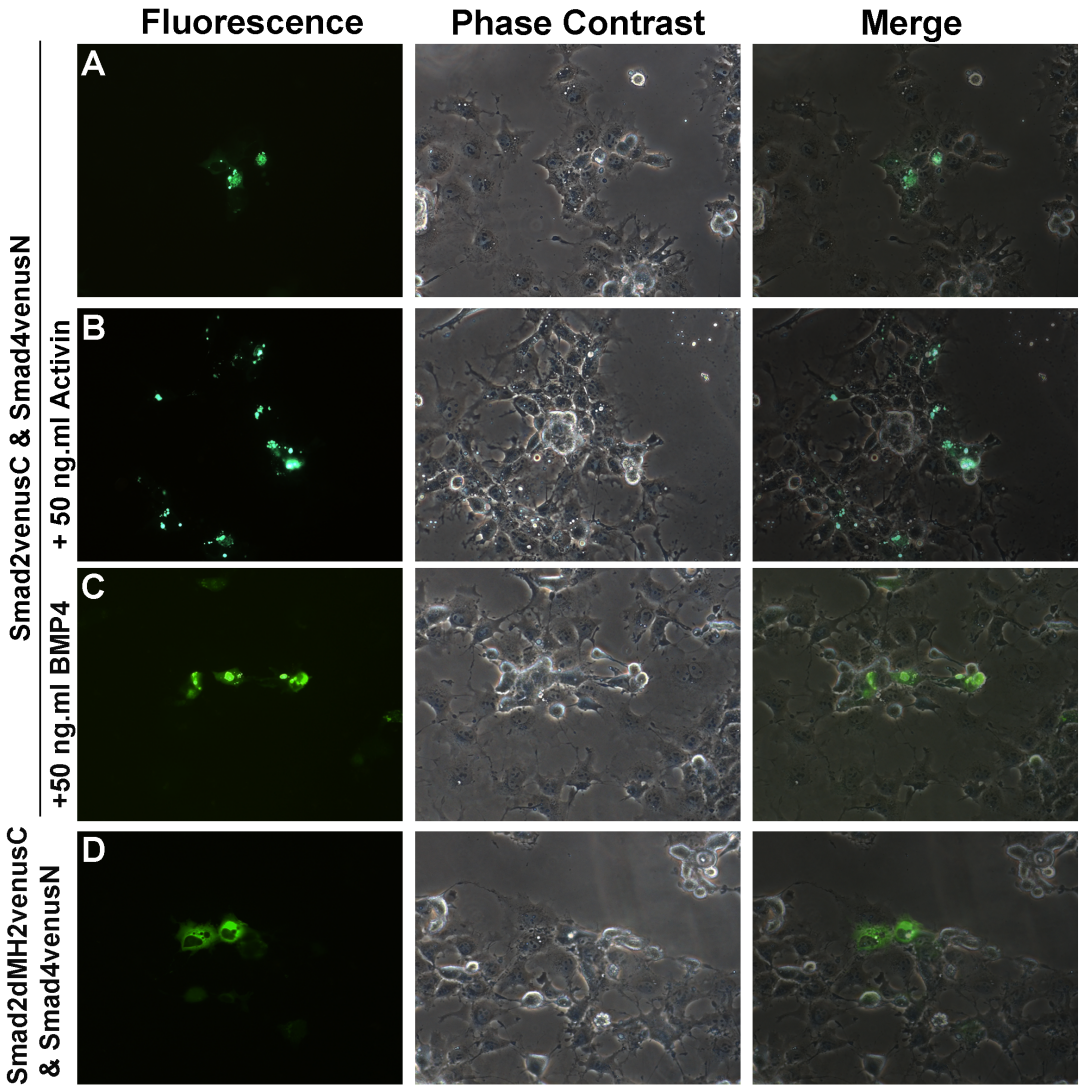

Fig. 4.7. Smad2-Smad4 BiFCo in vitro assays. COS cells were co-transfected with Smad2venusC and Smad4venusN plasmids and observed for fluorescence in time-course when cultured with or without TGFβ signalling proteins. An interaction was observed for Smad2venusC-Smad4venusN in normal medium conditions (A) and a similar level seen when cultured with 50 ng.ml Activin (B) and BMP4 (C). Fluorescence was also observed in a few cells (2-3) per well, between the negative control Smad2δMH2 construct and Smad4 (D).

What is the role of the SIP1- Smad1 interaction in neural fate acquisition?

Sip1, a member of the zinc-two finger homeodomain family proteins has been identified as a Smad-binding protein in yeast-two hybrid screens (Verschueren et al., 1999) and co-immunoprecipitation assays on cell culture extracts (Postigo, 2003; Postigo et al., 2003). Given that Sip1 is expressed in the neural plate (Sheng et al., 2003) and is induced by signals from the node at a time when cells become responsive to the level of BMP (Streit et al., 1998), it was proposed that a Sip1 may sensitize cells to the level of BMP signalling through an interaction with activated Smad1.

There are several methods for investigating protein-protein interactions, however bi-molecular interaction complementation assays provide a method for visualizing interactions in living cells (Hu et al., 2006) and therefore could provide a method for visualizing an interaction in neural tissue of developing embryos. However, despite transfection of SIP1 and Smad1 Venus fusion constructs into cell lines, electroporation in chick and microinjection in zebrafish embryos it was not possible to observe any positive interaction by this method. The differences observed in the sequence of *Xenopus* SIP1 with published sequence are unlikely to be responsible, as the amino acid variants are far from the reported Smad-interaction domain and are conserved in mouse and human sequences which have previously been shown to interact with Smad1 in co-immunoprecipitation experiments (Postigo, 2003; Postigo et al., 2003). It is possible that tagging of proteins with Venus protein fragments inhibits their ability by altering the proteins structural conformation thereby changing either their ability to interact with each other directly, or via inhibiting the recruitment of to co-factors/co-activators such as p300 (Postigo et al., 2003) that are required to mediate binding. Re-cloning of Sip1 to form an N-terminal conjugated fusion protein with the VenusC fragment or N- and and C-terminal fusions with VenusN may provide viable constructs for interaction

assays, however the long coding sequence of Sip1 (~ 3.5 kb) means that cloning of constructs is not trivial.

It's a possibility that COS cells do not have the correct complement of co-factors to enable an interaction to take place, however this would not account for the lack of interaction in chick or zebrafish embryos. Indeed, overexpression of positive control constructs in chick and zebrafish embryos, that did interact in COS cells in this study, did not provide observable levels of fluorescence, suggesting that insufficient levels of constructs were introduced into cells. It would be interesting nevertheless to attempt microinjection of constructs using capped mRNA in zebrafish, a method that has previously been used (Harvey and Smith, 2009), although this would require re-cloning of the interaction construct into a suitable vector.

Alternatively, a co-immunoprecipitation (co-IP) approach could be a valid method for investigating this interaction, although it would not enable the visualization of interactions in vivo. Such a method would require cloning of tagged constructs, which could be elctroporated into chick embryos and the ability for SIP1 and Smad1 to co-IP assessed in cells extracts from regions of the embryo including the neural plate, neural plate border assessed. Mutated constructs that are impervious to phosphorylation would enable investigation of whether BMP activation stimulates the interaction. Although the visualisation of the interaction between SIP1 and Smad1 was not possible by BiFCo assay in this study, what role an interaction may have in mediating a cells response to BMP signalling is still important to assess.

BMP signalling is involved in defining the border between the neural and non-neural ectoderm; high levels of BMPs and their target genes are expressed in the border region (Streit and Stern, 1999), and if BMP is inhibited in and around the edge of the neural plate, the border is shifted outwards (Streit and Stern, 1999; Linker et al., 2009), suggesting that BMP activity is crucial in positioning the border between neural plate and epidermis from which the neural crest and placodes arise. Interestingly, SIP1 is highly conserved and knockout mice (Van de Putte et al., 2003), and the knockdown of Sip1 in

zebrafish embryos (Delalande et al., 2008) show neural crest defects. Furthermore, mutations that lead to frame shifts or truncations causing a loss of one allele of SIP1 in humans, have been associated with Mowat-Wilson syndrome and Hirschsprung disease which are characterized by severe mental retardation and multiple neural crest defects (Amiel et al., 2001; Cacheux et al., 2001; Wakamatsu et al., 2001; Dastot-Le Moal et al., 2007). Thus, there is considerable evidence for a role for Sip1 in neural crest development, therefore an attractive hypothesis is that SIP1-Smad1 complex could be involved in the demarcation of the neural plate - epidermal boundary where the neural crest arise (Sheng 2003, Stern, 2006).

The consequence of an interaction between SIP1 and Smad1 could result in several possible outcomes, SIP1 could act to sequester Smad1, preventing it binding to Smad4 and retain it in the cytoplasm. Alternatively, SIP1 has been reported to act as either a repressor (Verschueren et al., 1999; Postigo et al., 2003; Sheng et al., 2003) or activator (Yoshimoto et al., 2005; van Grunsven et al., 2007) depending on the recruitment of co-factors, thus the cellular context is crucial for defining its mode of action. An interaction in the nucleus with activator co-factors could alter the function of one or both proteins into activators that could act at either BMP response elements or Sip1 binding sites.

It has been reported that there are several binding sites for Sip1 in the enhancer of the pan neural marker *Sox2* (Uchikawa et al., 2003), given the expression of Sip1 in neural tissue (Verschueren et al., 1999; Sheng et al., 2003; Delalande et al., 2008) and the loss of neural markers in Sip1 loss of function studies (Verschueren et al., 1999; Sheng et al., 2003; Van de Putte et al., 2003; Delalande et al., 2008) suggest that Sip1 acts positively to enhance *Sox2* in the neural plate and maintain neural fate. However, at the border of the neural plate where high levels of BMP and therefore phospho-Smad1 are present, the formation of a complex with Sip1 may help define not only the boundary between neural and non-neural fates, but also help define the border state, which would help explain the loss of neural crest fates in Sip1 loss of function studies (Sheng et al., 2003; Van de Putte et al., 2003;

Delalande et al., 2008) and human mutations (Amiel et al., 2001; Cacheux et al., 2001; Wakamatsu et al., 2001; Dastot-Le Moal et al., 2007).

Thus, Sip1 may have several functions during neural induction, firstly at the mid-line where its forms the neural-mesodermal boundary by inhibiting the expression of mesodermal genes in neural tissue (Sheng et al., 2003), as well as at the lateral border of the neural plate where it defines the neural – epidermal boundary by sensing the levels of BMP signalling through an interaction with Smad1.

Do Smad1 and Smad2 compete for binding of Smad4?

Neural tissue can be seen as having two boundaries; the lateral edge of the neural plate with the epidermis, and the mid-line of the embryo with mesoderm. Mesodermal and epidermal tissues that border the neural plate, are induced by signals involving Smad1 (BMP) and Smad2 (Nodal/Activin), respectively. Given that it has been proposed that Smad1 and Smad2 compete for binding to the common mediator Smad4 (Candia et al., 1997), it was proposed that might provide cells with a mechanism to sense relative levels of activated Smad1 and Smad2, thereby enabling cells to integrate signals from more than one pathway and make the choice between mesodermal, neural and epidermal fates.

To assess if there is a competitive interaction between Smad1 and Smad2 for binding to Smad4 it was proposed that a bi-molecular interaction assay may provide a means for visualizing these interactions and the inhibitory effect that stimulation of the opposing pathway may have. However, constructs transfected into COS cells showed non-specific interactions, including a mutated form of Smad1 (Smad1δMH2) with Smad4, furthermore Smad1 – Smad4, and Smad2 - Smad4 constructs were able to interact even in the presence of active signalling of the opposing pathway. Thus, this method was unable to provide a viable method for investigating a competitive interaction in this case.

The non-specific interactions between truncated proteins lacking previously identified interaction regions, means is impossible to say whether the interaction of the full-length protein reflects a specific interaction, thus, such negative controls are important for defining the fidelity of protein-protein interactions. However this is a limitation of such over-expression approaches, as these studies rely on tagged proteins acting with the specificity of the endogenous proteins, even though they may be introduced at far higher levels than seen endogenously. Whether the interaction of Smad1 and Smad2 with Smad4 is due to non-specific binding, in order to observe competitive inhibition of an interaction there has to be a dynamic change either caused by instability of the protein binding interaction or a short-half life of formed complexes as protein-protein interactions that are very stable would inhibit the utility of interaction assays such as BiFCo to visualize a dynamic competitive process. Additionally, cell culture conditions include serum that probably contains proteins able to stimulate TGFβ signalling, thus although cells were stimulated with the opposite pathway it is possible that a basal level of both BMP and Nodal pathways are stimulated meaning that both complexes continue to form.

Conclusion

In summary, bi-molecular fluorescence complementation assays were used to investigate the interactions between SIP1 and Smad1 and the proposed competitive binding of R-Smads with Smad4. However, this did not provide a valid method for visualizing these interactions and the methodology requires modification to improve its efficacy.

Chapter Five

The ground state of the pre-streak epiblast and the response of neural tissue to BMP inhibition.

Introduction

The earliest step in neural induction occurs in the pre-streak chick epiblast whereupon the first epoch of genes marking prospective neural plate development are expressed. It has previously been reported that, in vitro, explants of "lateral" epiblast tissue at this stage (mainly prospective non-neural ectoderm) can be induced to express neural markers in response to BMP antagonists (Wilson et al., 2000). Similarly, *Xenopus* ectodermal animal caps can be neuralised by BMP antagonists (Lamb et al., 1993; Sasai et al., 1994). Together these observations fit with the "default" model, which proposes that ectodermal cells are neuralised in response to BMP antagonists secreted by the organizer (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b; Harland, 2000). Thus evidence from both chick explants and *Xenopus* studies suggest neural induction depends ultimately on the level of BMP signalling.

In contrast, a model involving more complexity is emerging from experiments in vivo in the chick, where sequential steps and interacting signalling pathways including FGF signals are required (Streit et al., 2000; Linker and Stern, 2004; Stern, 2005; Papanayotou et al., 2008). No combination of BMP antagonists is able to induce any neural markers in the area opaca epiblast, a region competent to respond to neural inducing signals from the organizer (Streit et al., 1998; Linker and Stern, 2004). However, one set of experiments in the chick raised the possibility that not all of the ectoderm (as the default model predicts), but only cells close to the neural/epidermal border are sensitive to BMP and its antagonists (Streit et al., 1998; Streit and Stern,

1999).

This chapter aims to investigate this issue, by examining the effect of BMP inhibition on ectodermal tissue in the chick and *Xenopus*, to establish if they behave in a comparable way, and by investigating the status of specification of cells in the pre-streak epiblast, to establish whether the earliest step in neural plate development reflects a cell state similar to that of the neural plate border.

Results

The experiments in this section were done in collaboration with C. Linker and fully reported including methodology in Linker et al., (2009).

BMP inhibition induces neural plate border markers in chick

It was previously shown that BMP inhibition does not induce neural markers (*Sox3, Sox2*) in chick ectoderm (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004). However it has not been determined whether this treatment induces neural plate border markers (prospective neural crest/placodes). Electroporation of *Smad6* or *Smad7* into the area opaca epiblast induces *Pax7* (13/14; Figs. 5.1. A–C), *Dlx5* (9/9; Figs. 5.1. D–F), *Msx1* (9/10; not shown) and *Slug* (13/14; not shown) but not neural plate (*Sox2*: 0/23; Fig. 5.1. B), or mesoderm (*Brachyury*: 0/37;Not shown; Linker and Stern, 2004). It is possible that *Smad6* or *-7* alone do not inhibit enough BMP-activity for full neural induction. However, even a combination of *Smad6* + *Smad7* + dominant-negative-BMP-receptor (dnBMPR) + Noggin + Chordin + Cerberus, together with FGF and Wnt inhibitors, fails to induce neural markers (*Sox2*: 0/11; not shown). Thus, although BMP inhibition is insufficient for neural induction, it does induce neural plate border markers.

Expansion of the neural plate by BMP-inhibition requires cellular continuity of BMP-inhibited cells to the neural plate or its border

Studies using grafts of Chordin- or Noggin-secreting cells have shown that

inhibition of BMP affects neural/epidermal choice only at the neural plate border (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004). To test whether cell-autonomous BMP antagonists can reproduce this effect, we electroporated *Smad7*, *Smad6* or dominant-negative BMP-receptor (dnBMPR) as a line extending outwards from the prospective neural plate. These treatments cause a marked extension in the expression of *Sox2* and *Sox3* into the prospective epidermis and even into the extraembryonic area opaca (*Sox2*: 11/14 [*Smad7*; Figs. 5.2. A, B], 20/21 [*Smad6*; not shown], 4/5 [dnBMPR; not shown], 0/25 [GFP control; not shown]; *Sox3*: 7/7 [Smad7; Figs. 2C, D], 8/8 [Smad6; not shown], 0/21 [GFP control; not shown]). Expression of neural plate border markers is also dramatically extended (*Pax7*: 18/18 [Figs. 2E–G]; *Slug*: 11/12 not shown). Surprisingly, *Pax7* is not restricted to the *Smad*-electroporated cells (Figs. 5.2. G), but is also seen in neighbouring non- electroporated cells.

This last observation raises the possibility that cells from the host neural plate are stimulated to migrate laterally when BMP is inhibited. To test this, we compared cell movements between the electroporated side and the contralateral side (marked with DiI). No differences were observed between the two sides (not shown) (Linker et al., 2009), showing that the expansion of neural plate and border markers by misexpression of cell-autonomous BMP antagonists is due to induction rather than cell recruitment. Together, these results suggest that chick non-neural ectoderm cells can only be induced to express neural markers by BMP-inhibition when these cells form a continuous trail to the neural plate or its border. Without such continuity, only border markers are induced.

Cellular continuity with the neural plate or its border is necessary for neural induction by BMP-inhibition in *Xenopus*

Does *Xenopus* ectoderm respond in a similar way? It has been shown that BMP-inhibition is not sufficient to induce neural markers in prospective epidermis (descendants of the A4 blastomeres) and that neural markers are only induced in ventral epidermis by BMP- antagonists when FGF4 is also supplied (Linker and Stern, 2004; Delaune et al., 2005). A similar combination

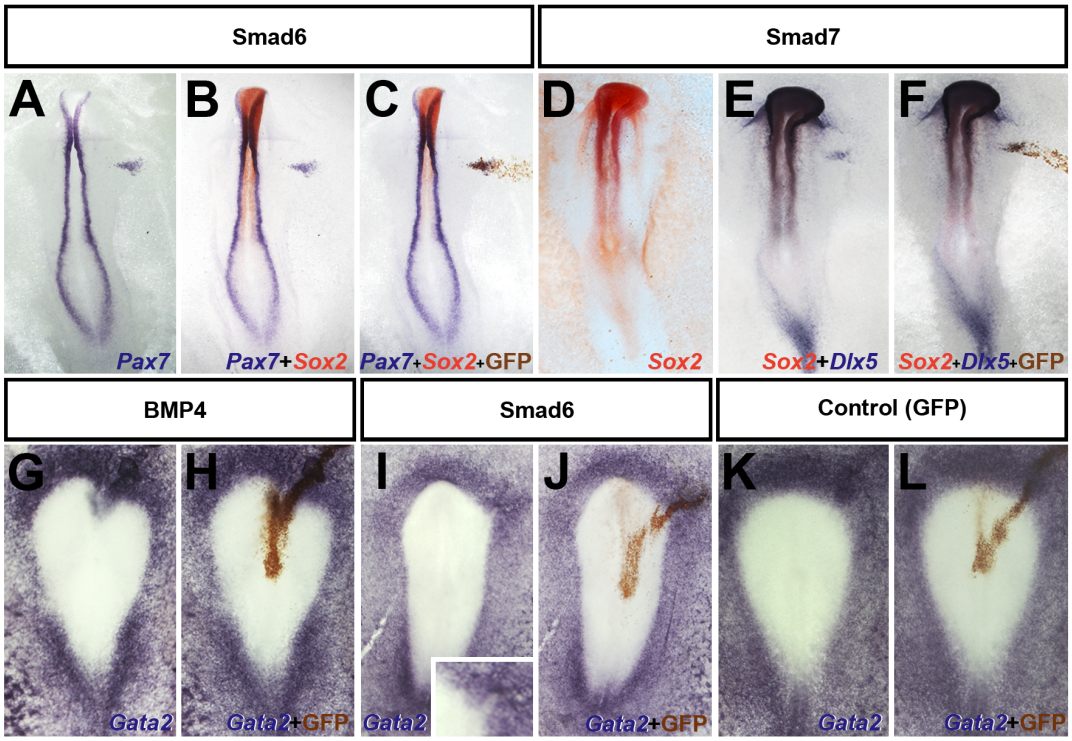

Fig. 5.1. BMP inhibitors induce neural plate border markers in chick. (A–L) Electroporation of *Smad6* or *Smad7* in prospective epidermis induces *Pax7* (A–C) and *Dlx5* (E, F) in the absence of *Sox2* (B, C and D, F). Electroporation of BMP4 induces *Gata2* in the neural plate (G, H). Inhibition of BMP by *Smad6* inhibits *Gata2* at the neural border (I, J). GFP (control) does not affect *Gata2* (K, L). (M–R) Electroporated cells were stained with anti-GFP antibody (C, F, H, J, L, for the embryos to their left).

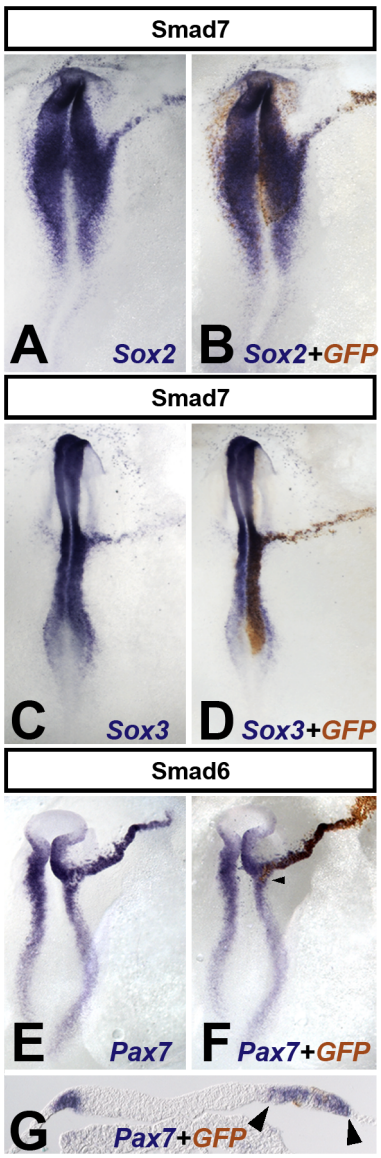

Fig. 5.2. Only the border of the neural plate is sensitive to BMP in chick. Electroporation of *Smad6* or *Smad7* as a line extending out from the neural plate induces an expansion in the expression of *Sox2* (A, B), *Sox3* (C, D) and *Pax7* (E, G). G is a section through the embryo in F (arrowhead), showing non-cell-autonomous expansion of *Pax7* (arrow- heads). Electroporated cells were stained with anti-GFP antibody (B, D, F and G for the embryos to their left).

(FGF4+*Smad6* or *Smad7*) in chick induces mesodermal markers (Linker and Stern, 2004), raising the possibility that the neural induction by this combination in *Xenopus* is indirect.

First, we confirmed our previous results: inhibition of BMP by injection of *Smad6* (1 ng) or *ΔSmad7* (10 pg) (Wawersik et al., 2005) does not induce neural markers when injected into the A4 blastomere (*Sox3* [*Smad6* 0/70; *ΔSmad7* 0/237] or *Sox2* [*Smad6* 0/60; *ΔSmad7* 0/ 324] (Figs. 5.3. A–D and not shown) Injection of a combination of *Smad6* (1 ng) or *ΔSmad7* (10 pg) and FGF4 (0.16 pg) in these blastomeres is now able to induce neural markers (*Sox3* [Smad6 108/120; ΔSmad7 85/103] *Sox2* [*Smad6* 46/71; *ΔSmad7* 81/ 102] Figs. 5.3. E–H, N, O for *Smad6* and not shown).

Next, we analysed whether neural induction by BMP inhibition and FGF activation requires mesoderm. We co-injected *Smad6* (1 ng) or *ΔSmad7* (10 pg) and FGF4 (0.16 pg) together with the nodal inhibitor CerS. To test the effectiveness of CerS, we injected CerS in the whole embryo (4 cells at the 4 cell stage, 1.5–2 ng). This inhibits the formation of mesoderm (*MyoD* 0/90, *chordin* 0/91, *brachyury* 0/102; not shown) and completely prevents gastrulation, as previously reported (Piccolo et al., 1999). We then tested whether inhibition of Nodal signalling and mesendoderm formation by CerS affects the induction of neural markers by BMP-inhibition+FGF4. Strikingly, co- injection of CerS + FGF4 + *Smad6* or *ΔSmad7* into one A4 blastomere strongly reduces the induction of *Sox3* (*Smad6* from 93% to 20.4%; n=212, Figs. 5.3 I, J and M or *ΔSmad7* from 82.5% to 6.5%; n=195) and virtually abolishes induction of *Sox2* (*Smad6* from 62% to 2.7%; n = 152, Figs. 3K–M; *ΔSmad7* from 79.4% to 1.4%; n=174). Together, these data suggest that in *Xenopus* embryos, as in the chick, the induction of neural markers by FGF4 and BMP antagonism is indirect, due to either a prior induction of mesendoderm or to cooperation with Nodal signalling (see also (De Almeida et al., 2008).

To determine whether the activity of FGF4 is due to its mesendoderm-inducing ability, we examined whether FGF8a (an isoform without mesoderm inducing activity; Fletcher et al., 2006) can induce neural markers when

injected in combination with BMP inhibitors into ventral epidermis. First, to test the effectiveness of FGF8a, 10–50 pg were injected into one cell at the two-cell stage. This did not affect the expression of a mesodermal marker (*Brachyury* 0/60; Fig. 5.4. A), but did expand neural markers (*Sox3* 18/23 not shown, *β-tubulin* 25/28; Fig. 5.4. B), as expected (Fletcher et al., 2006). Next, we tested the effects of injection of FGF8a (10–50 pg) into the A4 blastomere: neither mesodermal nor neural markers were induced (*Chordin* 0/40*, β-tubulin* 0/17, *Sox2* 0/6, not shown, *Sox3* 0/30; Figs. 5.4. C, D), as was reported for FGF4 (Linker and Stern, 2004; Delaune et al., 2005). We then tested if co-injection of FGF8a (10–50 pg) + *Smad6* (1 ng) can induce neural markers in ventral epidermis: neither neural (*Sox2* 0/24 not shown, *Sox3* 0/23; Figs. 5.4. E, F), nor mesodermal markers (*Chordin* 0/31, not shown) were induced. These results strengthen our previous suggestion that induction of neural markers by FGF activation and BMP antagonism is an indirect consequence of mesendoderm induction.

We then analysed whether BMP-antagonists induce border markers in ventral epidermis in *Xenopus*, as shown above for chick embryos. Indeed, injection of *Smad6* into the A4 blastomere induces the neural border markers *Pax3* (20/26; Figs. 55 A–C), *Slug* (62/73; Figs. 5.5. D–F), *Hairy2A* (22/33; Figs. 5.5. G–I) and *Xiro1* (17/19; not shown), but not neural markers (Sox2, *Sox3*; Figs. 5.3 A–D). Thus, as in chick, BMP inhibition in *Xenopus* ventral epidermis induces neural plate border markers.

Finally, we examined if the border of the *Xenopus* neural plate is especially sensitive to BMP-inhibition, as it is in chick. Injection of *Smad6* into the prospective neural plate border (blastomeres A2/3) causes lateral expansion of *Sox3* (43/45; Figs. 5.5. J, L; white brackets in J and black arrows in L) and *Slug* (38/42; Figs. 5.5. M, N). These results in chick and *Xenopus* show that although border markers can be induced by BMP-inhibition in lateral/ventral epidermis, neural induction in the same cells requires the BMP-inhibited cells to form a continuous trail to the neural plate and/or its border.

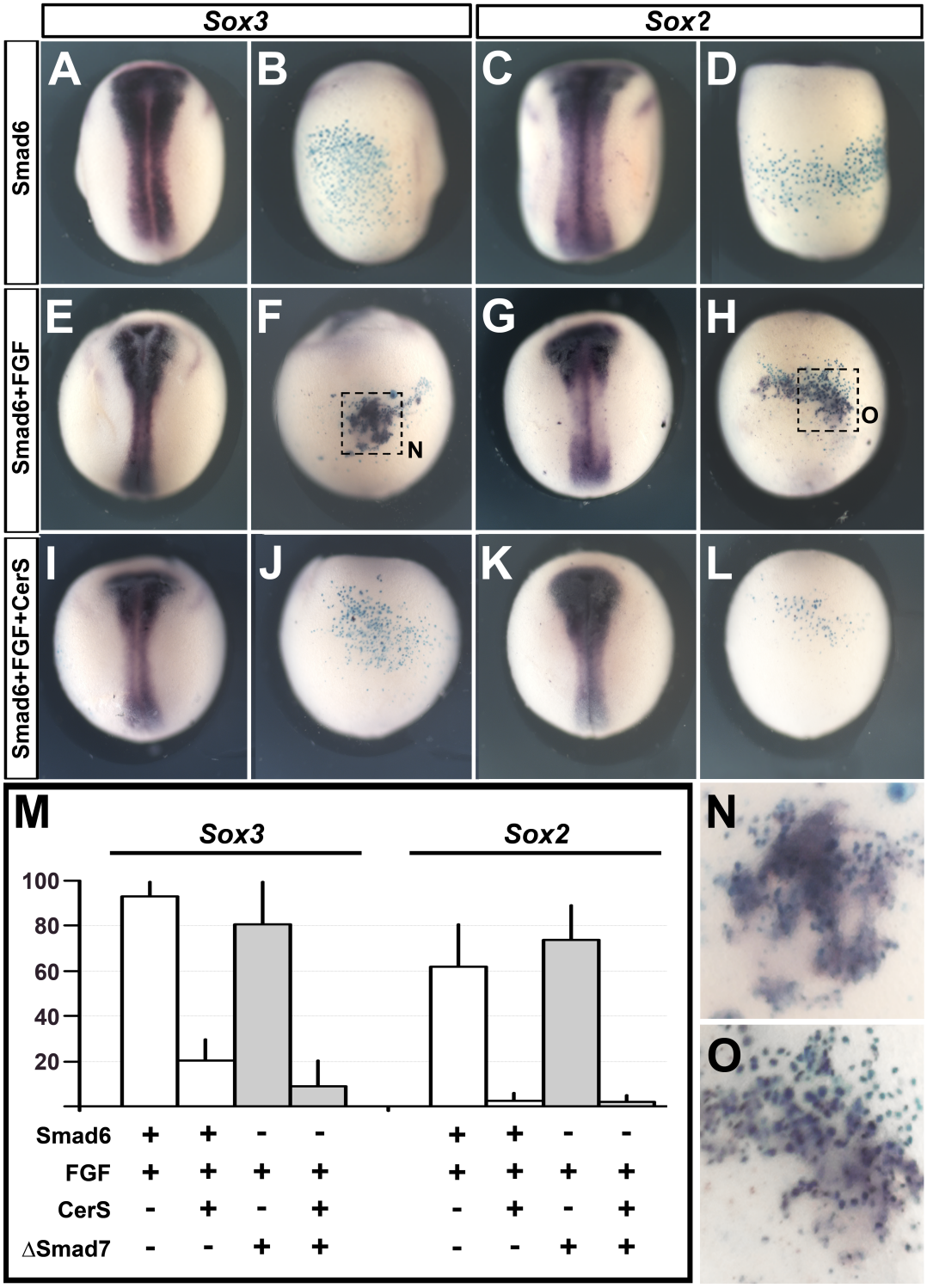

Fig. 5.3. BMP inhibition together with eFGF (FGF4) activation induces neural marker expression indirectly in *Xenopus*. (A–L) Inhibition of BMP by injection of *Smad6* into the A4 blastomere does not induce either *Sox3* (A, B) or *Sox2* (C, D) expression. FGF4 together with BMP inhibition into the A4 blastomere induces *Sox3* (E, F, N) and *Sox2* (G, H, O). Neural induction by

the former combination is inhibited when Nodal signalling is blocked: injection of *Smad6* + FGF4 together with CerS no longer induces *Sox3* (I, J) or *Sox2* (K, L). (M) Quantification of *Sox3* and *Sox2* expression in the different experiments described above. A, C, E, G, I and K dorsal views. B, D, F, H, J and L ventral views of the embryos to their left. N and O are enlargements of the areas enclosed by a square in F and H, respectively.

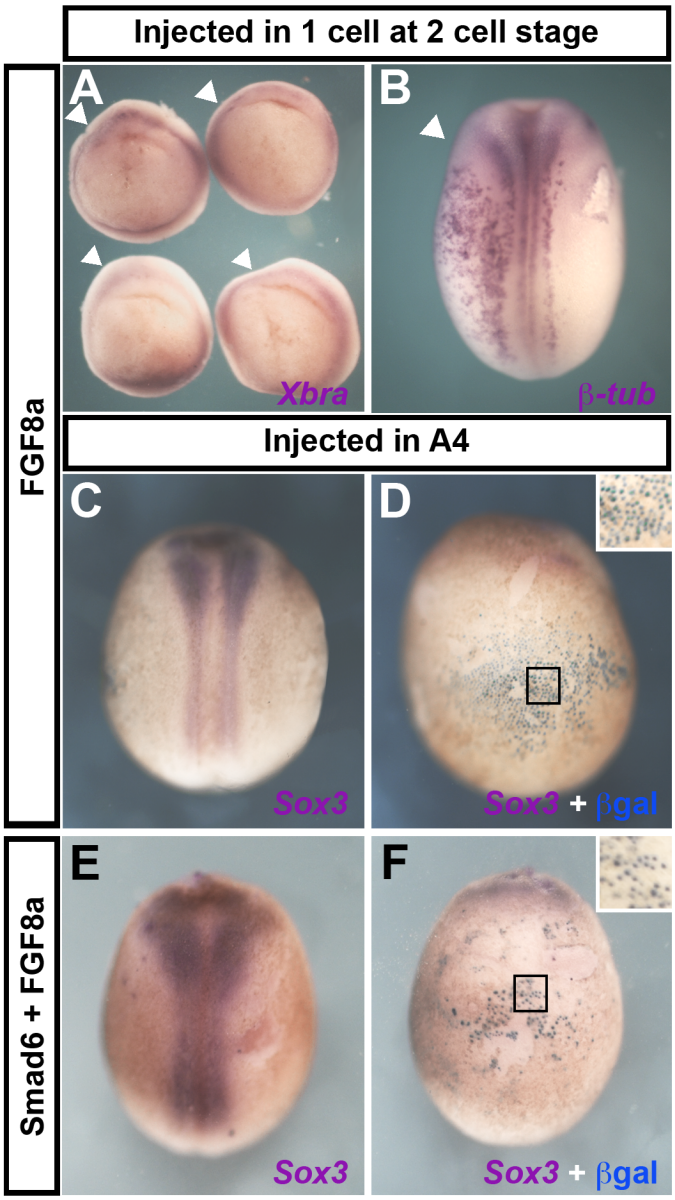

Fig. 5.4. BMP inhibition together with FGF8a does not induce neural marker expression in *Xenopus*. (A–B) Injection of FGF8a into one cell at the two-cell stage does not alter *Brachyury* expression at the gastrula stage (A) but does expand β-tubulin expression at the neurula stage (B); arrowheads indicate the injected side. (C–F) Injection of FGF8a into an A4 blastomere, alone (C, D) or in combination with the BMP inhibitor *Smad6* (E, F) does not induce *Sox3* expression in ventral epidermis. A: vegetal view; B, C and E are dorsal views; D and F are ventral views of the embryos to their left. Black squares show the area enlarged in the inset in panels D and F.

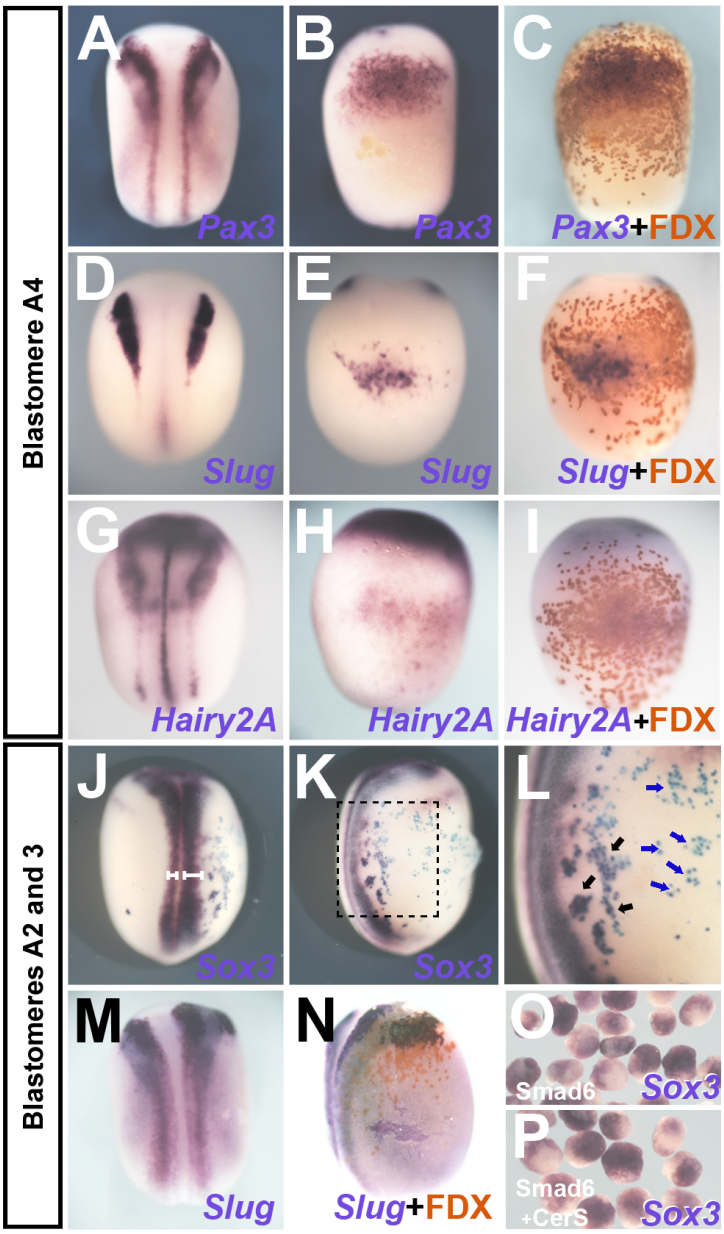

Fig. 5.5. Only the border of the neural plate is sensitive to BMP inhibition in *Xenopus*. (A–I) *Smad6* (1 ng) injection into the A4 blastomere induces *Pax3* (A–C), *Slug* (D–F) and *Hairy2A* (G–I). (A, D, and G: dorsal view; B, C, E, F, H and I: ventral view of the embryo to their left). (J–N) Injection into blastomere A2/3 expands *Sox3* (J–L) and *Slug* (M, N). J, M: dorsal view; K, L and N are lateral views of the embryos to their left. White brackets in J show the extension of the neural plate in the injected and non-injected sides of the embryo. The black square in K indicates the area enlarged in L. Black arrows in L point to injected cells adjacent to the endogenous neural plate, expressing *Sox3*; the blue arrows point to injected cells distant from the

endogenous neural plate, which do not express *Sox3*. Injected cells were recognized by FDX or LacZ (C, F, I, K, L and N, for embryos to their left). (O–P) Animal caps from *Smad6*-injected embryos at the 2-cell stage express *Sox3* (O), which is not inhibited by CerS (P).

The *Xenopus* animal cap behaves like a neural plate border and contains prospective border cells

The above results are at odds with the widely reported finding that *Xenopus* animal caps, thought to contain cells destined to contribute to epidermis but not neural tissue, can be neuralised easily by BMP antagonists (Harland, 2000; Muñoz-Sanjuán and Brivanlou, 2002; De Robertis and Kuroda, 2004; Vonica and Brivanlou, 2006). We therefore performed animal cap assays: animal caps were isolated at stage-8 from embryos injected with *Smad6* in the animal pole at the 2-cell- stage. Unlike injections into A4, animal pole injections of *Smad6* induce *Sox3* (Fig. 5.5. O; 38/38). Moreover, co-injection of *Smad6*+CerS does not inhibit *Sox3* induction in animal caps (Fig. 5.5. P;P 50/53). This confirms that animal caps can be neuralised by BMP-antagonism and that this is insensitive to Nodal signalling.

The observation that BMP-inhibited cells can express neural markers if they form a continuous trail to the neural plate or its border, together with the fact that animal caps are easily neuralised by BMP antagonists, prompted us to test whether animal caps contain prospective neural plate or border cells. To this end, we assessed the contribution of animal cap cells to the neural plate and the neural/ epidermal border by fate mapping animal caps. Donor embryos were injected with fluorescein-lysine dextran (FDX) in both cells at the 2-cell stage, and the animal cap excised from these embryos at stage 8. The excised tissue was grafted into an identical region of unlabelled host embryos at the same stage and analysed at stage-19, examining both fluorescence as a lineage tracer and expression of the neural marker *Sox3* (Fig. 5.6. A–C). The outlines of all small and all large transplants, at stage 8 and stage 19, were drawn in separate model embryo outlines (see Materials and Methods; Fig. 5.6. D, E and H, I). In Fig. 5.6 F and J (stage 8) and G and K (stage 19), the areas that receive a cellular contribution from 60%, 80% and 93% of the transplants are shown in red, orange and yellow, respectively. At stage 19, the region expressing *Sox3* is also shown (grey; Fig. 5.6. G, K). 60% of even the smallest caps (Fig. 5.6. D–G) contribute to the anterior neural plate itself and virtually all caps (80%) contribute to the anterior neural/epidermal border (prospective placodes; Figs. 5.6. D–K). These data

show that nearly all animal caps dissected at stage 8 contain neural plate and/or neural plate border cells which correlates with the *Xenopus* fate map (Dale and Slack, 1987).

Chick epiblast explants behave like the *Xenopus* animal cap.

It has been reported that explants of "lateral" chick epiblast (mainly prospective non-neural ectoderm) can be induced to express neural markers in response to BMP antagonists in culture (Wilson et al., 2000, 2001). The above results raise the possibility that chick explants are equivalent to *Xenopus* animal caps and are specified as border cells. To assess this we dissected "medial" and "lateral" epiblast (as defined by Wilson et al., 2000, 2001) from stage-XII (Eyal-Giladi and Kochav, 1976) chicken embryos and assessed expression of neural, neural border and mesodermal markers after 40 hours' culture.

Both medial and lateral explants express neural (*Sox3*: medial 17/18, lateral 20/24; *Sox2*: medial 18/19, lateral 16/18 Fig. 5.7. C-D'), and neural border markers (*Dlx5*: medial 14/20, lateral 13/17, *Msx1*: medial 11/17, lateral 18/24; *Gata3*: medial 16/20, lateral 13/17; *Slug*: medial 12/22, lateral 16/26; *Pax7* medial 11/16; lateral 11/16; *Six4*: medial 8/12; lateral 10/15; *Eya2*: medial 8/11; lateral 7/13 Fig. 5.7. H-N') and *ERNI* which is a marker of both the early pre-neural state and the border of the neural plate (medial 8/9, lateral 10/12 Fig. 5.7. O, O'). However, they do not express later neural or mesodermal markers (*Sox1*: medial 0/15, lateral, 0/13; *Tbx6*: 0/12; 0/9 Fig. 7 B, B', G, G'). These results suggest that under these conditions, epiblast explants from any embryonic region of the pre-streak stage embryo are specified as neural plate border explaining the discrepancy between the results of BMP inhibition in vivo (Streit et al., 1998; Streit and Stern, 1999b; Linker and Stern, 2004; De Almeida et al., 2008) and in vitro (Wilson et al., 2000, 2001).

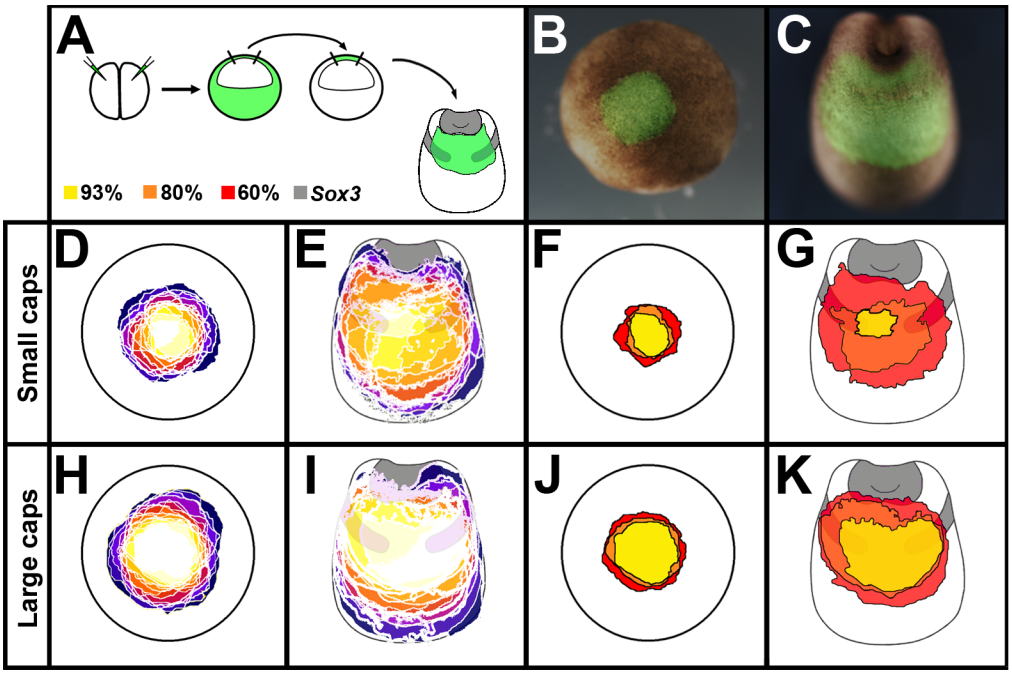

Fig. 5.6. The *Xenopus* animal cap contains cells fated as anterior neural border. (A) Caps from FDX-injected embryos were obtained at stage 8, transplanted to uninjected hosts and analysed for *Sox3* at stage 19. (B) Example of a transplant at st.8 after 1.5 h healing, the same embryo at st.19 (C). (D–K) Results of all small (D–G; n=14) and large (H–K; n=15) transplants, each in a different colour, at stages 9 (D, H, F, J) and 19 (E, G, I, K; including Sox3 expression). In D, E and H, I, the regions of overlap are shown in progressively lighter shades, with white indicating a region where all transplanted caps overlap. In F, G and J, K, the areas that receive a cellular contribution from the transplant are in Yellow: 93%; Orange: 80%; Red: 60%.

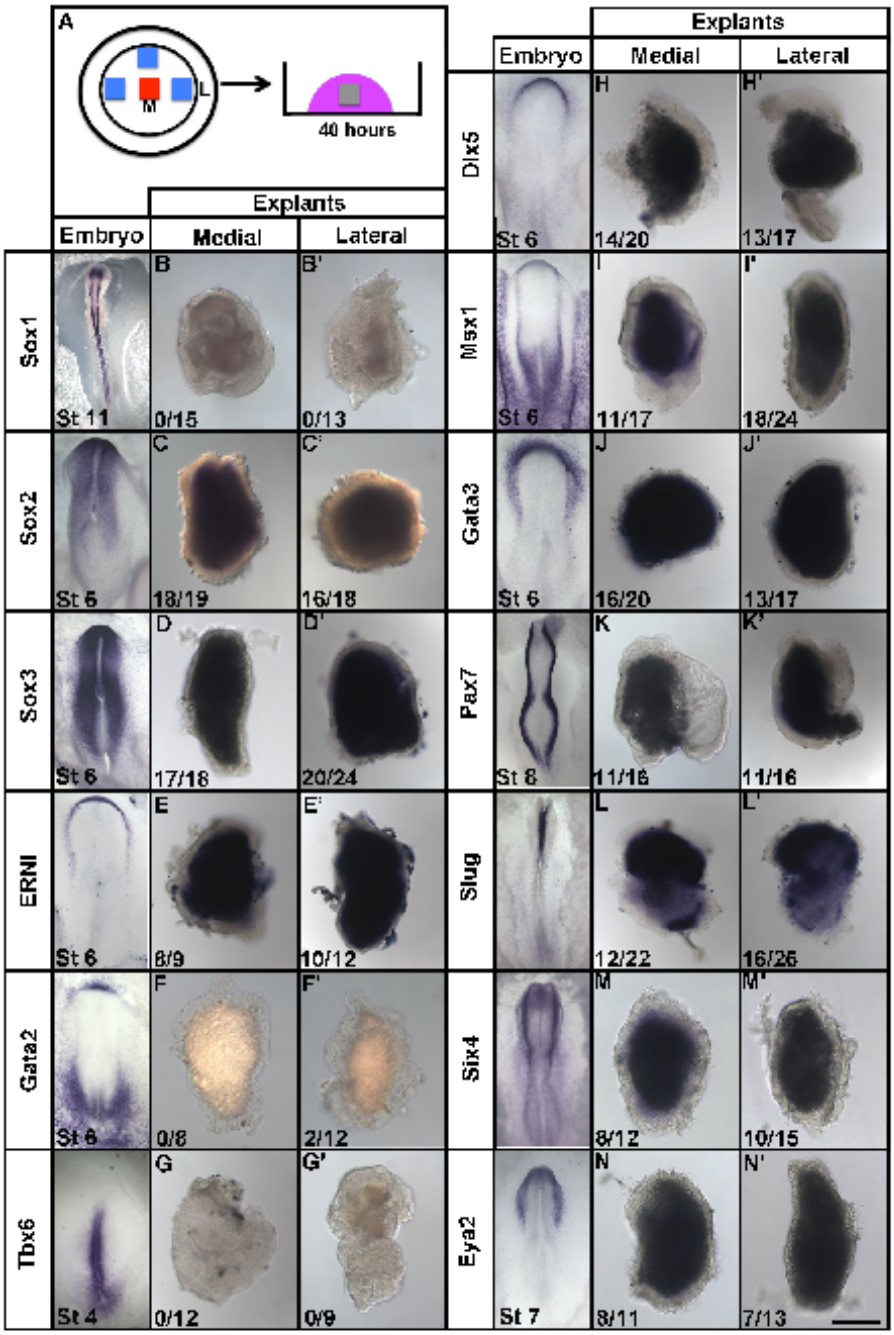

Fig. 5.7. Chick explants express neural plate and border markers. Medial or lateral stage-XII explants (A) were analysed for neural plate, border, epidermis and mesodermal markers (B-N) All express neural: *Sox2*, *Sox3*, and border markers: *ERNI, Dlx5, Msx1, Gata3, Pax7, Slug Six4 Eya2,* but not epidermal: *Gata2,* or mesodermal: *Tbx6*, markers. Scale bar in N' (100 μM) applies to B-N'.

MAPK signalling is required for neural plate border specification in chick explants.

One difference between the *Xenopus* animal cap and the chick explant assays is that neural markers are only expressed in the latter in the absence of BMP inhibition. Standard culture conditions for chick explants require a defined culture medium that includes N2 supplement, which contains a number of factors including insulin, that are intended to promote neural differentiation, whereas *Xenopus* animal caps are cultured in simple saline. It has previously been reported that FGF signals (which signal through MAPK) are required for expression of neural markers in cultured chick explants (Wilson et al., 2000), however insulin-like growth factor signalling can also signal through a similar pathway involving MAPK. Could signals from the culture medium be responsible for the neuralisation of chick explants? To assess this we tested the effects of added insulin-like growth factors (IGF) and of pharmacological inhibitors of FGFRs (SU5402) or MAPK (U0126) on "medial" and "lateral" epiblast explants (Wilson et al., 2000, 2001) from stage-XII (Eyal-Giladi and Kochav, 1976) chicken embryos, assessing the expression of the neural and neural border markers *Sox2* and *Dlx5* after 40 hours' culture.

Explants cultured in N2 supplement lacking insulin were severely impaired, and the few that survived (5/42) were unusable for assessment of marker expression. Explants from both regions cultured in medium supplemented with N2 with 1 μM SU5402 showed weak expression of neural but not border markers (*Sox2*: medial 4/6, lateral 3/10; *Dlx5*: medial 0/5, lateral 0/4, Fig. 5.8. B-C'), but expression of both markers was lost at ≥ 2 μM SU5402 treatment (*Sox2*: medial 0/24, lateral 0/16; *Dlx5*: medial 0/10, lateral 0/15 Fig. 5.8. D-G'). Similarly, explants from both regions cultured with 1 μM U0126 weakly expressed *Sox2* (medial 3/3, lateral 2/2 Fig. 5.8. H-H') but not *Dlx5* (medial 0/5; lateral 0/4 Fig. 5.8. G-G'), but no expression was seen in explants from either region at 2 μM or 5 μM (*Sox2*: medial 0/14, lateral 0/8; *Dlx5* medial 0/16, lateral 0/8 Fig. 5.8. L-M'). Expression was not inhibited by control U0124 treatment (*Sox2*, medial 12/13, lateral 13/15: *Dlx5* medial 15/18, lateral 15/21

Fig 5.8. N-S'). Finally, 50 nM IGFI could rescue the loss of both markers by 2 μM SU5402 treatment in explants from both regions (*Sox2*: medial 5/7, lateral 9/12; *Dlx5*: medial 6/8, lateral 7/9, Fig. 5.8. T-U'), confirming that the lack of expression is not due to non-specific effects of the inhibitor. These results show that IGF is required for the survival of explants in culture, and suggest that although FGF signalling is required for expression of neural and border markers, IGF may be able to compensate for FGF through MAPK.

Chick explants behave like the anterior border, pre-placodal region in extended culture.

The anterior border region of the neural plate contains a horse-shoe-shaped domain containing the progenitors that give rise to the paired sensory placodes of the head; rostrally cells gives rise to the otic placode, with prospective lens, epibranchial and andenohypophyseal cells situated progressively posterior to this (Streit, 2007). It has been reported (Bailey et al., 2006) that if cells of any region of this, pre-placodal region (PPR) in the stage 6 chick are isolated from the embryo and cultured, irrespective of their position, they express markers of the lens placode. This suggests that the ground-state of specification of cells at the anterior neural plate border is lens. The observation that epiblast from stage XII-chick embryos, responds to BMP in a similar way as the neural plate border (Wilson et al., 2000), and expresses markers which are expressed exclusively in the pre-placodal territory (*Eya2* and *Six4*) (Fig 5.7. M-N'), raises the question of whether the cultured tissue enters an anterior border, pre-placodal-like state? If so, cells should share the ground-state of differentiation seen in the PPR (Bailey et al., 2006). To investigate this we dissected "medial" and "lateral" epiblast (Wilson et al., 2000, 2001) from stage-XII (Eyal-Giladi and Kochav, 1976) chick embryos and assessed expression of specific markers of the lens and otic placodes, and for lens development after either 40 hours' or 6 days' culture.

Both medial and lateral explants express lens specific markers often confined to a small region of the tissue after 6 days (*δ-crystallin*; medial, 12/33, lateral, 25/53: *L-maf;* medial 9/32, lateral 12/34 Fig. 5.9. D, E, H, I), but not after 40 hours of culture (*δ-crystallin;* medial 0/39, lateral, 25/53: *L-maf:* medial 0/28,

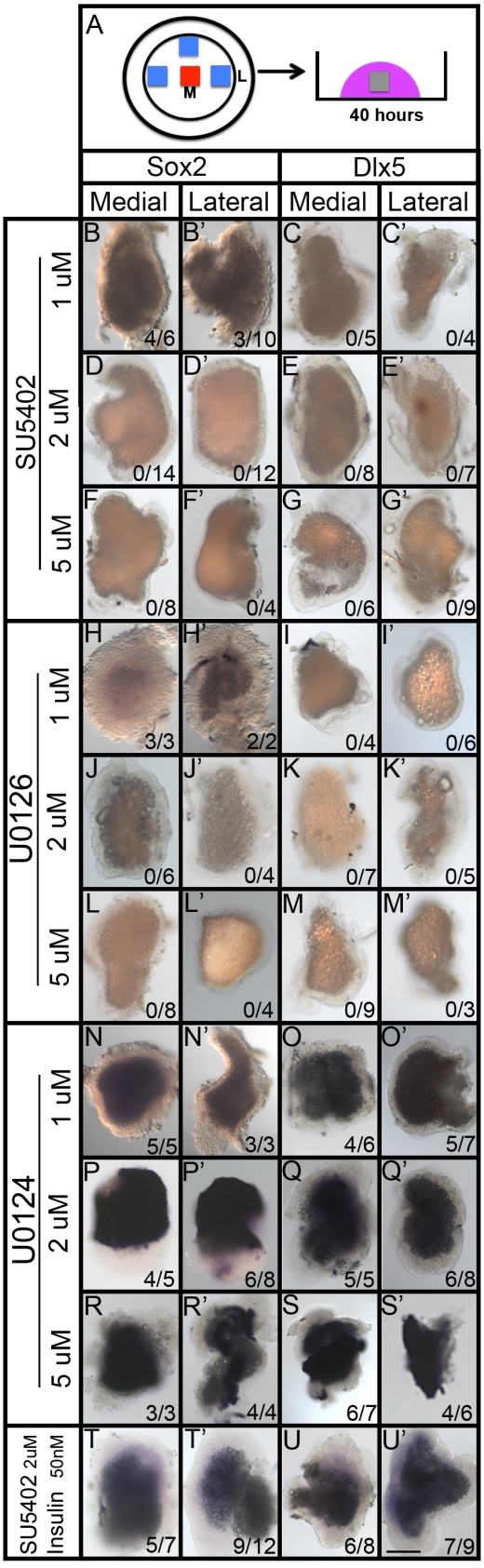

Fig. 5.8. IGF can rescue loss of FGF signalling in culture chick explants

Fig. 5.8. IGF can rescue loss of FGF signalling in culture chick explants. Medial or lateral stage-XII explants (A) were analysed for their expression of neural (*Sox2*) and neural plate border (*Dlx5*) markers when treated with chemical inhibitors of the FGF and MAPK pathway. Weak expression of Sox2 was seen in explants treated with 1 μM SU5402 (B, B') but lost at higher concentrations (D, D', F, F'). SU5402 treatment abolished Dlx5 expression (C,C', E, E', G, G'). Inhibition of MAPK signalling by U0126 abolished expression of both *Sox2* and *Dlx5* (H-M'), but was expressed control treatments with U0124 (N-S'). 50 nM of IGF added to explants treated with SU5402 was able to rescue to expression of both markers (T-U'). Scale bar in U' (100 μM) applies to B-U'.

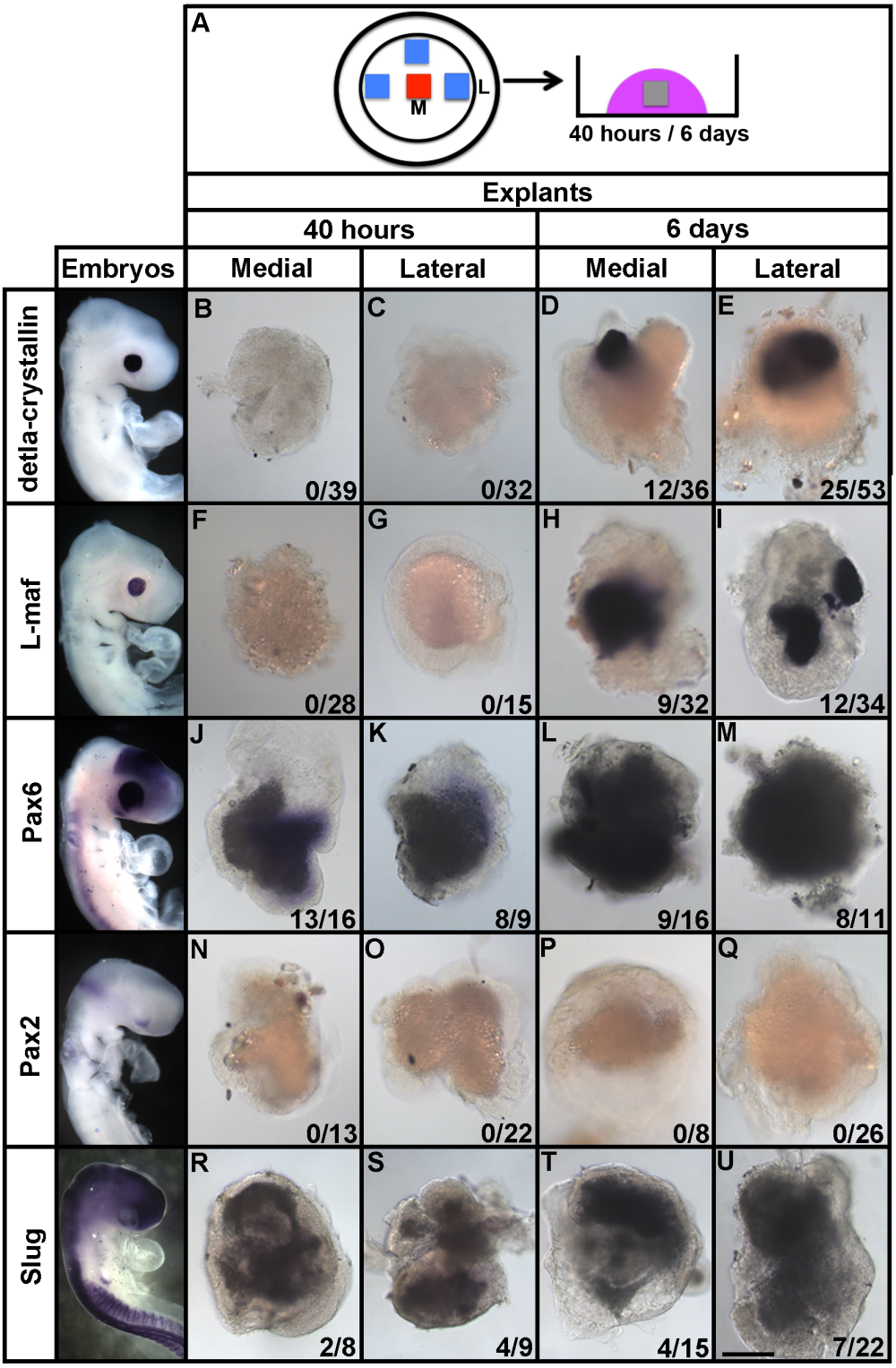

Fig. 5.9. Chick explants express lens markers in extended culture.

Fig. 5.9. Chick explants express lens markers in extended culture. Medial or lateral stage-XII explants (A) were cultured for 40 hours or 6 days and assessed for their expression of lens, otic and neural crest markers. Both expressed the specific lens markers *δ-crystallin* and *L-maf* after 6 days (D,E, H, I), and *Pax6* a marker of the PPR required for lens specification after 40 hours and 6 days (J-M), but not the otic marker *Pax2* (N-Q). Explants express neural crest markers at both 40 hours and 6 days (R-U). Scale bar in U (100 μM) applies to B-U.

lateral 0/15 Fig. 5.9. B, C, F, G). *Pax6* which is required for lens specification in the PPR and in the lens placode is expressed after both 40 hours (*Pax6*: medial 13/16, lateral 8/9 Fig. 5.9 J, K) and 6 days culture (*Pax6*: medial 9/16, lateral 8/11 Fig. 9 L, M). But the otic marker *Pax2* is not expressed after either 40 hours (*Pax2*: medial 0/13, lateral 0/22 Fig. 5.9. N, O) or 6 days (*Pax2*: medial 0/8, lateral 0/26 Fig. 5.9. P, Q). Lentoid-like like structures normally have a lens like vesicle structures, however whilst sections on *δ-crystallin*+ve explants showed a spherical region of expression (not shown) the strong signal occluded the visualisation of structural aspects of the explants. These results suggest that under these conditions, epiblast explants are ultimately specified as lens, confirming that cells act like the anterior neural plate border pre-placodal region and share its ground state of specification.

What do the *δ-crystallin* negative explants express?

The observation that epiblast explants can express lens specific markers suggests that their ground state of differentiation is similar to that of the PPR. However, the finding that between ~ 28-47% of explants express lens specific markers raises the question of what the remaining explants become. One possibility is that they develop as neural crest, another derivative of the neural border. To assess this, we dissected “medial” and “lateral” epiblast (Wilson et al., 2000, 2001) from stage-XII (Eyal-Giladi and Kochav, 1976) chicken embryos and assessed expression of neural crest markers after either 40 hours' or 6 days culture. 26-31% of explants expressed neural crest markers after 6 days (Slug; medial 4/15, lateral 7/22 Fig. 5.9. R-U). These results suggest that the reason why some of the explants do not express lens markers, or indeed express lens markers like *δ-crystallin* throughout the explants, is they are neural crest, a tissue that has been reported to inhibit the development of lens in the ectoderm (Bailey et al., 2006).

Do all prospective cells pass through a border-like phase and ultimately have a default fate of lens?

Prior to gastrulation the chick epiblast expresses the first group of neural response genes (Streit et al., 2000)(Chapter 3), in culture this tissue has a default fate of neural plate border and ultimately lens. These observations

raise the question of whether all early prospective neural cells share a similar cell state? To investigate whether this is a general characteristic of cells differentiating along the neural lineage, a protocol for deriving 9N2 chick embryonic stem (ES) cells towards a neuronal lineage was used to assess for the expression of border markers (*Pax6, Eya2*) and lens markers *(δ-crystallin*) in time course.

9N2 chick embryonic stem cells can be differentiated towards neuronal fates under defined culture conditions.

The 9N2 chick embryonic stem (ES) cell line (Petitte and Yang, 1997) has been previously shown to be able to give rise to cells from all 3 germ layers (Acloque et al., 2001), and like the epiblast, cannot contribute to the germ line. Chick ES cells do not act in culture like those from mouse or humans, for example they do not spontaneously form embryoid bodies upon removal of LIF (Petitte et al., 2004), the timing of their differentiation in culture is slower and no efficient protocol for deriving neural cells exists (Lavial and Pain, 2010). A variety of culture protocols exist for the derivation of neuronal cells in mouse ES cells. Therefore, modified protocols were explored to provide a methodology for exploring the phases of neural differentiation. To assess if 9N2 cells can be directed towards neural fates, three 21-day protocols and two shorter protocols of 4 and 8 days, were assessed by immunohistochemistry for their ability to support expression of the neuronal markers Neurofilament and Transitin (Nestin) in cultured cells.

All three 21-day protocols (4 days retinoic acid, 0, 2 or 4 days treatment with growth factors (SHH, FGF8, GDNF), and culture up to day 21 with a neurobasal medium) (Fig. 5.10. A 3-5) gave rise to cells with neuronal-like morphology, which co-labelled with Neurofilament and Transitin (Fig. 5.10. A 1-3). As observed for differentiation cultures in other species (Nishikawa et al., 1998; Yamashita et al., 2000; Ying et al., 2003a; Lowell et al., 2006), cells in these cultures were highly heterogenous, many cells were undifferentiated and did not have a neuronal morphology and were Neurofilament and Transitin negative. The shortened 8-day treatment (4 days retinoic acid, 4 days growth factors) also gave rise to cells that co-labelled with Neurofilament

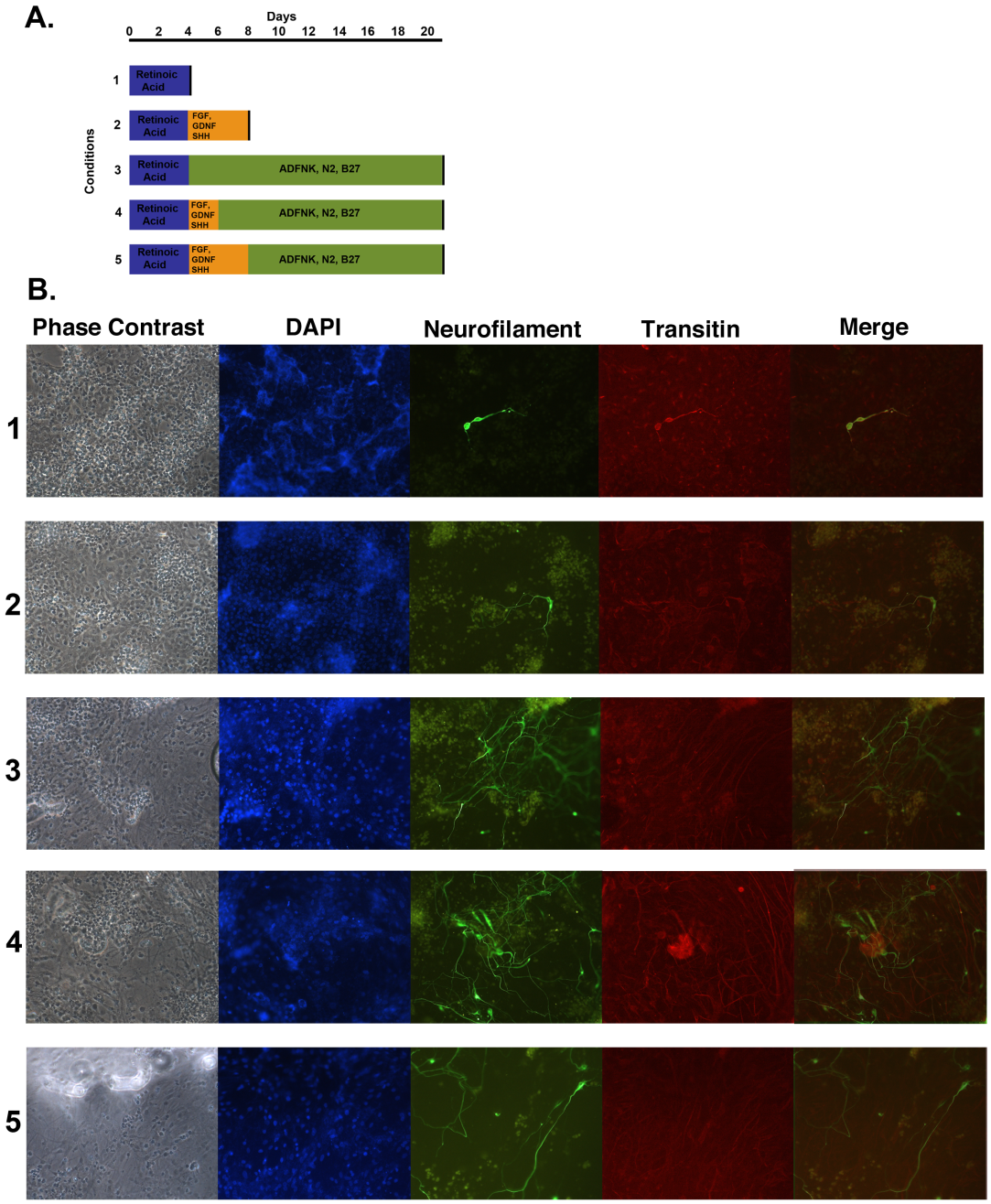

Fig. 5.10. Chick 9N2 embryonic stem cells can be directed to a neuronal fate in defined medium conditions. 9N2 ES cells were differentiated under 5 sets of neuralising conditions (A) and assayed for neuronal markers (neurofilament, NF and transitin, Trans) by immunohistochemistry. Conditions 1 and 2, 4 day RA and 4 days retinoic acid + 4 days growth factors, gave rise to very few NF+ve/Trans+ve cells and they had comparibly stunted axonal processes. Conditions, 3, 4 and 5 all 21-day protocols gave rise to 50-200 NF+ve/Trans+ve cells and which showed long axonal processes. In all treatment conditions cell cultures remained heterogenous.

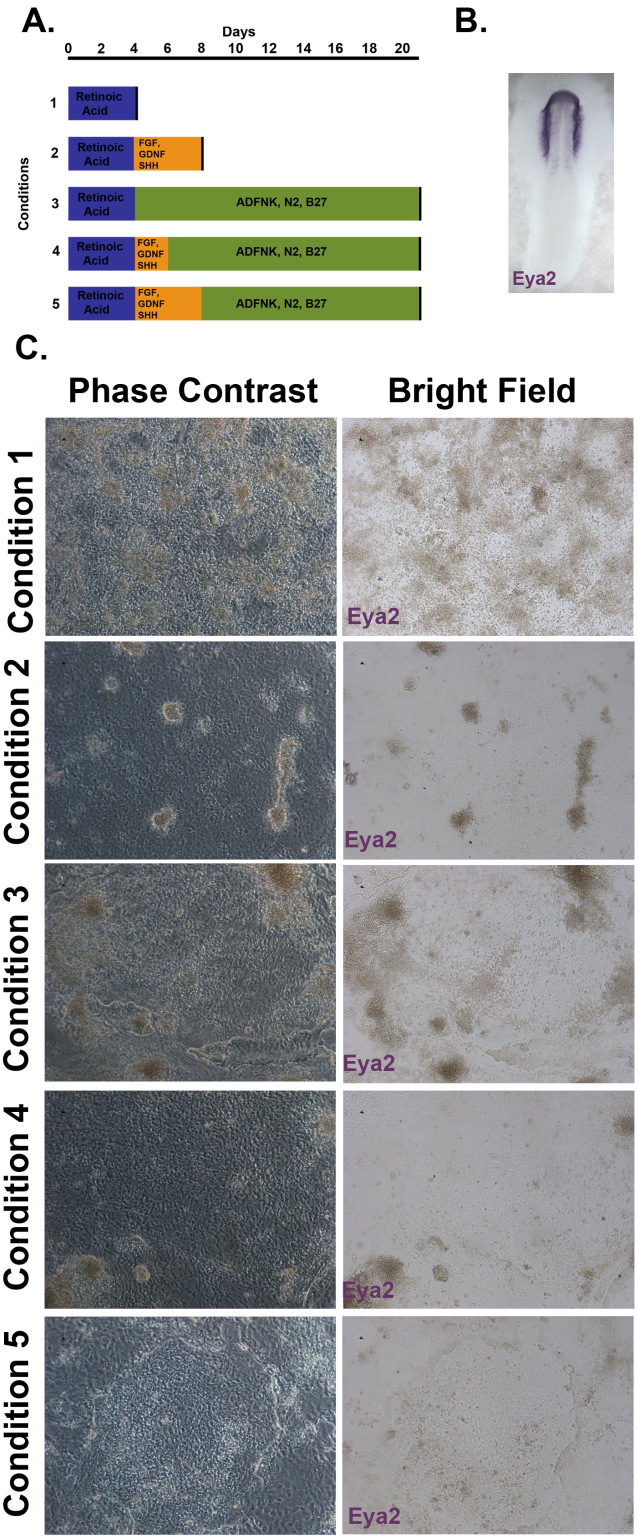

Fig. 5.11. 9N2 cell do not express the anterior neural border marker *Eya2* when cultured under neuralizing conditions. Chick 9N2 embryonic stem cells were differentiated in neutralizing conditions (A) and assessed for the expression of the PPR marker *Eya2* by in situ hybridization (B). No expression of *Eya2* was observed under any of the culture conditions.

and Transitin but had less pronounced morphology (Fig 5.10. B, Condition 2) and cells had shorter axonal projections. Finally, in 4-day retinoic acid treated cultures, only 1-2 cells per well labelled with Neurofilament and Transitin, and had stunted neuronal-like morphology. These results suggest that all protocols provide conditions that can direct 9N2 cells to the neural lineage, with longer, 21-day treatments providing the most effective conditions for deriving neuronal like cells.

Do 9N2 chick embryonic stem cells express markers of the neural plate border and the lens under neuralising conditions?

Given that these protocols provide a methodology for neuralising multipotent 9N2 chick ES cells, the question of whether they share the default fate of specification of the chick epiblast can be now be addressed. To assess if cells pass through a phase similar to that of the neural plate border and ultimately lens, three 21-day protocols and two shorter protocols of 4 and 8 days were assessed for their ability to induce border markers (*Eya2* by in situ and Pax6 by immunohistochemistry) and the lens specific marker, δ-crystallin (δ-CRYST) by immunohistochemistry.

No expression of *Eya2* was observed for 9N2 cells after 4, 8 or 21-day treatments (Fig 5.11. B), despite correct expression in positive control embryos (Fig 5.11. A). Similarly, no PAX6 or δ-CRYST expressing cells were observed in 9N2 cells after 4, 8 or 21 day treatments, despite antibodies labelling cells in positive controls consisting of frozen sections through embryos at stage 15 (not shown). Thus at these time points under these treatment conditions cells do not express border or lens markers.

Do 9N2 ES cells pass through a phase of PAX6 expression within 4 days of retinoic acid treatment?

Given that some 9N2 cells had neuronal-like morphology and stained for neuronal markers (Neurofilament and Transitin) after only 4 days of retinoic acid treatment, could a neural border like phase occur earlier in the cells? To assess this, 9N2 cell cultures were treated for 1 - 4 days with retinoic acid and assessed for the presence of PAX6 and Transitin proteins.

No PAX6 positive cells were observed in 9N2 cell cultures treated for 1 – 4

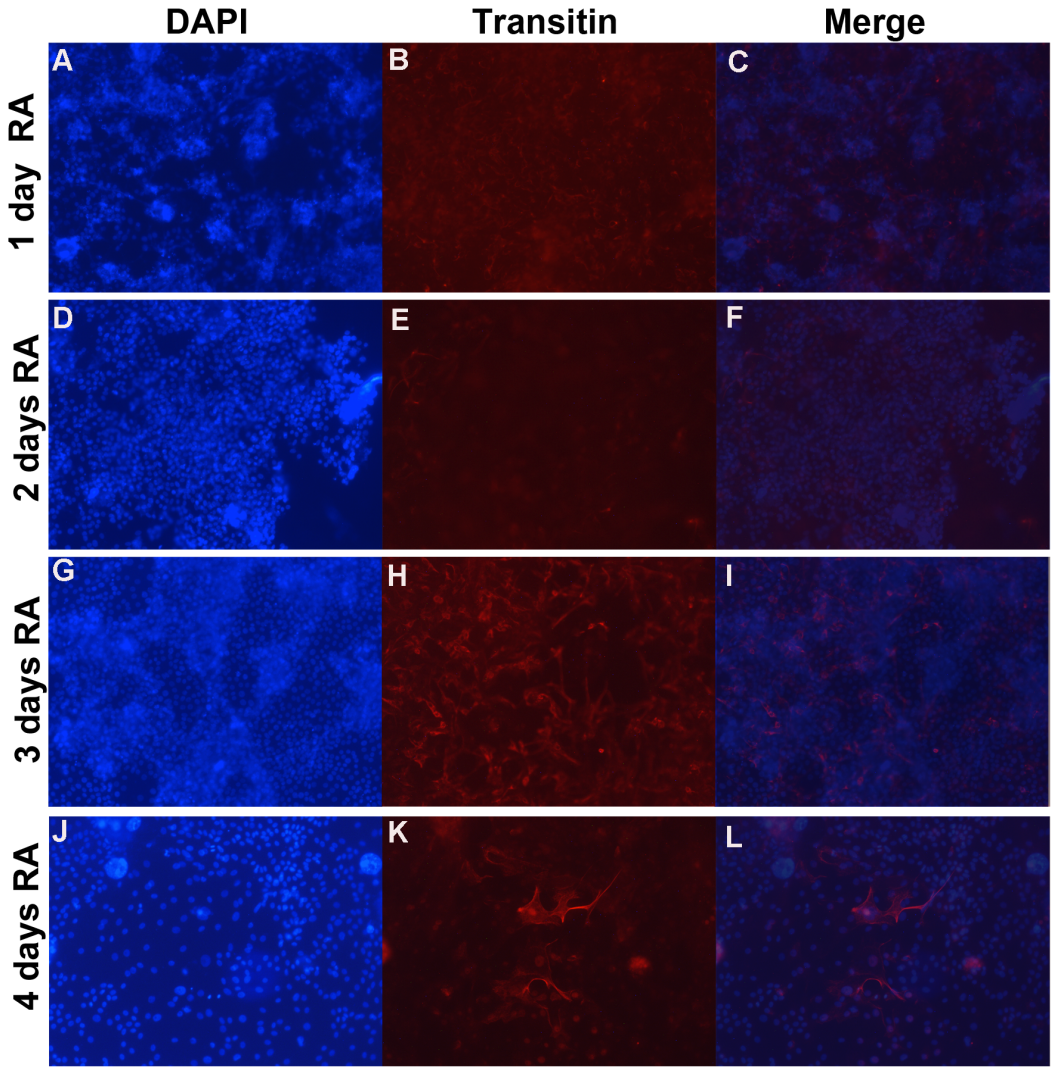

Fig. 5.12. Chick ES cells treated with retinoic acid for 4 days have glial-like properties. Chick 9N2 ES cells were treated for 1-4 days with retinoic acid and assessed for the Transitin (Trans) by immunohistochemistry. After 1 day Trans+ve cells were observed in cultured cells (B), as the time of exposure increased cells differentiated and a population of Trans+ve cells (E,H,K) had a neural crest, glial-like morphology (D-L).

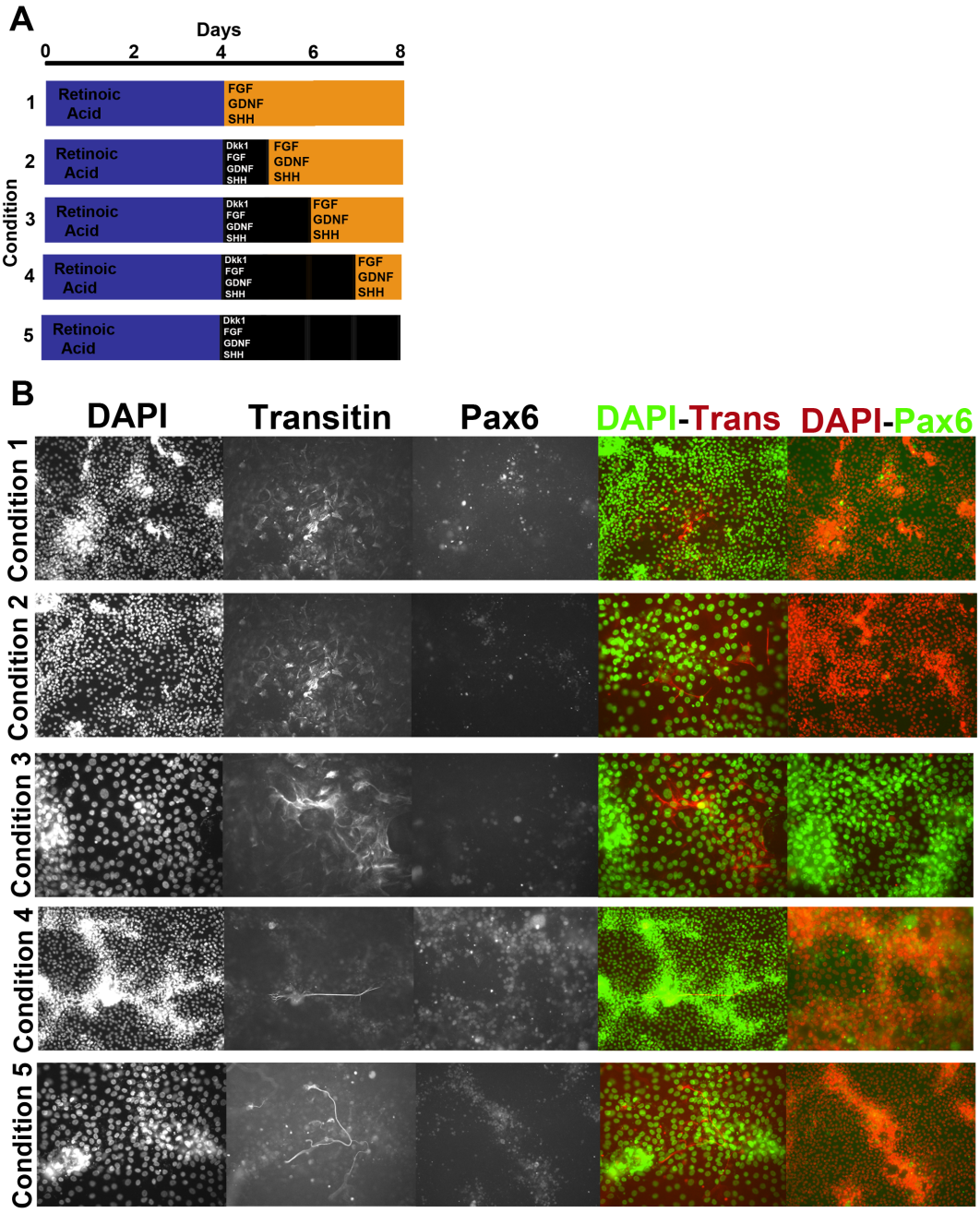

Fig. 5.13. Wnt inhibition can neutralize chick embryonic stem cells after 8 day culture. Chick 9N2 ES cells were treated for increasing periods with the Wnt antagonist *Dkk1* after an initial treatment of 4 days with retinoic acid (A). Whilst transitin positive cells were observed in conditions lacking DKK1 and in exposure for 1 day (1,2) pronounced morphology of neuronal cells was observed in cultures treated for 2-4 days with Dkk.

days with retinoic acid (not shown), but many cells in these cultures label with Transitin, and had a neural glial like morphology which became more pronounced with increased exposure to retinoic acid (Fig 5.12. A-M). These observations suggest that retinoic acid treatment pushes cell towards to a neural crest fate, as although Transitin is expressed in neural and glial precursors, the lack of PAX6 expression is suggestive of non-neural, non-placodal cells.

Could Wnt inhibition promote an anterior neural-border fate in 9N2 cell culture protocols?

It has been reported that Wnt activity regulates cell fate choices at the neural plate border; active Wnt signalling is required to induce neural crest tissue (Trainor and Krumlauf, 2002; Li et al., 2009; Steventon et al., 2009), whilst inhibition of Wnts are required for cells of the preplacodal region (Litsiou et al., 2005; Ohyama et al., 2006; Schlosser, 2006). Therefore, given the observation that treatment of chick ES cells with retinoic acid promotes differentiation towards a cell population expressing Transitin but not PAX6, subsequent inhibition of Wnt signalling might provide a protocol for deriving anterior neural border fates by shifting cells between border fates. To investigate whether Wnt inhibition can promote pre-placodal fates in ES cells, 9N2 cell cultures were treated for a total of 8 days: 4 days with retinoic acid followed by 4 days with growth factors (FGF8, SHH, GDNF) in combination with 1-4 days of the Wnt inhibitor Dickkopf (Dkk1). Additionally cells were treated with 4 days retinoic acid followed by 4 days of growth factors as a control for the effect of Wnt inhibition. Cells were assayed for PPR and glial markers (PAX6 and Transitin).

No PAX6+ve cells were observed in 9N2 cell cultures after 8 days of treatment involving 1-4 day treatment with Dkk1 (not shown). Many Transitin positive cells with glial-like morphology were seen in cells cultured without Dkk1, and in cultures treated with 1 or 2 days exposure to Dkk1 (Fig. 5.13.). However, Transitin expressing cells with a neuronal like morphology were observed in cultures that had received 3 or 4 days of Dkk1 treatment (Fig 5.13.). These results suggest that Wnt inhibition by Dkk1 may enhance a neuronal fate in these cultures, however this level of treatment is not sufficient

to block formation of glial-like (Transitin-positive, PAX6-negative) population of cells in these cultures.

Discussion

Experiments mainly from *Xenopus* led to the default model, which states that BMP inhibition is the only signal needed for neural induction (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b; Harland, 2000). However, experiments mainly from the chick have lead to considerable debate about whether this mechanism is sufficient to explain neural induction (Streit and Stern, 1999; Stern, 2005; Stern, 2006). BMP inhibitors alone cannot induce any neural plate markers (even very early pre-neural markers like *Sox3*) in vivo to date (Streit et al., 1998; Linker and Stern, 2004). The current view from chick experiments is that additional factors are required, and in particular that FGF signalling mediates the early responses to neural induction (Streit et al., 2000; Sheng et al., 2003; Stern, 2006; Gibson et al., 2010; Pinho et al., 2011). Several experiments in the chick have suggested that, in vivo, only the border of the neural plate can respond to BMP signals (Streit et al., 1998; Streit and Stern, 1999). However, cells of the pre-streak chick epiblast, a time that marks the onset of the first epoch of neural response genes (Streit et al., 2000; Pinho et al., 2011), can respond to BMP signalling (Wilson et al., 2000). This raised the question of whether only the neural plate border can respond to BMP signals in the ectoderm and whether in vitro the early epiblast has a ground state that is similar to that of the border.

BMP-inhibited cells require cellular continuity with the neural plate and its border to express neural markers.

Here we show that in vivo, BMP inhibition induces border markers in non-neural ectoderm of both chick and *Xenopus* embryos, but cells will only express neural markers if the BMP-inhibited cells form a continuous line with the cells of the neural plate and/or its border. These findings suggest that BMP inhibition enables neuralising factors to spread outwards from cells in contact with the neural plate ("homeogenetic induction", or induction of neural plate by neural plate; (Mangold, 1929; Mangold, 1933; Nieuwkoop et al.,

1952; Servetnick and Grainger, 1991). Over-expression of BMP inhibitors in cells adjacent to the neural plate or its border would allow these cells to respond to homeogenetic neural inducing signals emanating from the neural plate, resulting in an expansion of the neural territory. Thus in the context of BMP misexpression experiments only cells in the ectoderm, receiving both BMP inhibition and homeogenetic signals from the neural plate will be neuralised. Hence why tissue far from neural plate in the ventral ectoderm of *Xenopus* or distal epiblast (despite being competent to remake neural tissue) do not express neural markers in response to BMP inhibition alone.

However, these results are somewhat paradoxical; BMP inhibition in ectoderm far from the neural plate causes this prospective epidermal tissue to express neural border markers but not neural markers, whilst BMP inhibition of neural border cells induces neural markers. Even an increase the amount of BMP inhibition (in the case of chick even by a combination of *Smad6* + *Smad7* + dnBMPR + Noggin + Chordin + Cerberus, together with FGF and Wnt inhibitors) outside the border is still not sufficient to induce neural markers. This suggests that the border markers induced by BMP inhibitors alone does not represent a full border state, and that additional factors and maintenance signals perhaps from underlying mesoderm are also required for full neural crest specification (Streit and Stern, 1999; Steventon et al., 2009).

Given the fact that cell contact with the neural plate and its border are required for BMP inhibited cells to express neural markers (Fig. 5.2), the best candidate this cellular communication effect is the Notch pathway. Notch has been implicated in establishing the border of the neural plate (Kintner, 1992; Cornell and Eisen, 2002; Endo et al., 2002; Glavic et al., 2004), in generating boundaries between adjacent domains in many other systems and in synchronizing neural differentiation events in ES cell populations (Lowell et al., 2006).

The animal cap behaves like the neural plate border and contributes cells to it.

Similar to the neural plate border, *Xenopus* ectodermal animal caps can be easily neuralised by BMP antagonists (Lamb et al., 1993; Sasai et al., 1994).

Therefore, we explored whether animal caps might contain some neural plate and/or border cells. Fate mapping animal caps of a range of sizes revealed that even small caps contribute cells to the anterior neural plate itself in as many as 60% of cases, and nearly all caps contribute to the prospective placodal domain at the border of the anterior neural plate. Furthermore, isolated animal caps express both anterior neural (*Otx2*) and border (XAG1/XCG1) markers (Lamb et al., 1993; Knecht et al., 1995; Lamb and Harland, 1995). Although these findings are consistent with previous fate maps made at the 32-cell stage (Dale and Slack, 1987; Moody, 1987), they demonstrate that virtually all stage 8 animal caps excised contain cells fated to become neural plate border. These findings may help explain why animal caps can be neuralised so easily by BMP-antagonists.

Thus, animal cap explants contain prospective border cells (as well as prospective neural plate in many cases). This implies that when animal cap assays from BMP-antagonist-injected embryos are used for assessing neural induction, the animal cap preserves cellular continuity between the prospective neural plate/neural plate border and prospective epidermis, through which neural inducing signals can spread (see above). This may also explain why neural marker expression is always restricted to a subset of cells in animal caps excised from BMP-inhibited embryos.

The state of specification of the pre-streak chick epiblast is neural, neural border and ultimately has a ground state of lens.

The first step in neural plate development in the chick corresponds to the pre-streak epiblast (Streit et al., 2000; Pinho et al., 2011), however unlike in later development this prospective neural tissue is responsive to BMP signalling (Wilson et al., 2000). Given the importance of the neural plate border as a source of cells responsive to BMP, this led us to investigate the status of specification of the pre-streak epiblast. Culture of early epiblast explants showed that the entire epiblast initially enters a state similar to that of the neural plate border, explaining why it is able to respond to BMP signalling. This is consistent with fate maps that reveal that almost the entire epiblast contributes cells to the neural plate and/or its border (Rudnick, 1935; Rudnick, 1938; Hatada and Stern, 1994). Furthermore, this is supported by their

subsequent differentiation into lens, which is the ground-state of differentiation in the anterior neural plate border (Bailey et al., 2006).

These findings are in contrast to a previous study that suggested the early epiblast is already patterned into neural and epidermal regions (Wilson et al., 2000). Although the studies were carried out under the same conditions, part of the reason for these differences is in the selection and interpretation of the markers used. For example, *Msx1* was interpreted in the previous study as a marker of non-neural ectoderm (Wilson et al., 2000), even though it is expressed highly at the border of the neural plate (Fig 5.7). Thus, whilst the expression of similar markers, including *Pax6*, *Sox2*, *Msx1* (Wilson et al., 2000), were confirmed here, the expression of a larger range of markers, including additional markers of the neural plate border, enabled us to identify the neural, neural border as the specification state of cells from anywhere in the epiblast.

The finding that cells from anywhere in the epiblast have a ground state of the neural plate and its border is also consistent with the expression pattern of the pre-streak neural genes *Sox3* and *ERNI* in the chick (Streit et al., 2000). Both are expressed broadly in the epiblast, a finding consistent with the observation that FGF signalling from the hypoblast layer, which comes to completely underlie the epiblast, can induce these genes (Streit et al., 2000). Furthermore, the expression pattern of *ERNI* (Streit and Stern, 1999; Streit et al., 2000) and *Msx1* in the early epiblast and later at the border of the neural plate suggests that the initial state of the epiblast is border-like. Furthermore, it is interesting to speculate that these markers may reflect the multipotent state of both the epiblast and the border tissues.

The finding that genes expressed in the neural plate are also expressed in cultured epiblast tissue could reflect the fact that a proportion of explants acquire a neural plate state in culture. However, whilst *Sox2* and *Sox3* are expressed throughout the neural plate they also overlap with cells at the border, thus their expression could reflect the border state.

What is the role of culture conditions?

One difference between the *Xenopus* animal cap and the chick explant assays is that neural markers are only expressed in the latter. One possible reason for this difference is that chick explants are grown in the presence of a culture medium that includes N2 supplement, containing a number of factors including insulin, which is intended to neuralise tissue. As previously reported FGF signalling is required in explants for the expression of neural markers (Wilson et al., 2000), however, the finding that epiblast does not survive culture in the absence of insulin means that it is difficult to investigate its effect directly. IGF can rescue loss of FGF signalling by SU5402 treatment, suggesting that it may augment the neuralising role of endogenous FGF signalling. However, whilst the culture conditions may not provide a means for investigating the signals involved neural induction, they do provide conditions to explore the state of cells.

Is lens the ground state of all prospective neural tissue?

The finding that epiblast explants pass through a border-like phase and ultimately express lens markers suggests that the early epiblast is in a similar state of specification as the endogenous border. Thus, even though cells bordering the neural plate contribute to all placodal fates in a rostral-caudal progression (Bhattacharyya et al., 2004; Streit, 2007), the initial state of specification of cells from any region of the PPR is lens (Bailey et al., 2006). It could be argued that given that both studies use similar explant culture conditions, these may be responsible for determining the direction of differentiation in culture. However, if neural crest is ablated in vivo, then ectopic lens tissue is formed in a region of the cranial ectoderm suggesting that the crest secretes inhibitors preventing cells in the ectoderm from the default fate of lens (Bailey et al., 2006).

The finding that a similar number of explants express neural crest markers as express lens markers in extended culture, could also argue against a default of lens. It could be that the numbers between lens- and crest-expressing explants are similar because they reflect the same population of explants – which would explain why *δ-crystallin* expression is confined to a small region

(Fig 5.9. B-B'), or that there is a partial overlap in these numbers, with some explants having a ground state exclusively of neural crest, some pre-placodal, some both, and some neither. Nevertheless, *δ-crystallin* is a specific marker of the lens and its expression in explants in extended culture, suggests that a proportion of cells at will differentiate towards the lens fate.

Given these findings, do all early prospective neural cells enter a similar border-like phase and ultimately share the ground-state of specification? We approached this question by using chick ES cells that, similar to the early epiblast, are multipotent progenitors that can give rise to all three germ layers, but cannot contribute to the germ line. Furthermore, they express markers similar to the epiblast, including *ERNI*. Thus the differentiation of ES cells towards neural fates could provide an assay to test whether the earliest step in neural induction is the induction of a pre-neural-border state in prospective neural cells. However, although culture conditions including retinoic acid, FGF, SHH and neurobasal medium did lead to formation of neuronal-like cells from ES cells, no expression of markers indicative of the anterior border or lens were observed. However, cells with glial-like morphology in ES cells treated for 4 days with retinoic acid suggests that this treatment stimulates cells to express Transitin (Fig 5.12) but not PAX6 or Eya2 (Fig 5.11). Given that Wnt has been shown to regulate cell fates at the border of the neural plate, treatment with Wnt inhibitors, which enhance the number of neuronal-like cells, may provide conditions for investigating this issue. However, the timing of differentiation of cells in culture is difficult to correlate with events in the embryo, any further studies would require a complete time-course to establish if ES cell share the ground-state of differentiation of the lens.

Conclusion

In summary these experiments suggest a hitherto unknown importance of the neural border cell-state, which is important not only for interpreting BMP misexpression studies in *Xenopus* and chick, but also unexpectedly is the ground state of the epiblast at the start of the neural induction cascade which ultimately has a ground-state of lens.

Chapter Six

General Discussion

BMP inhibition & The Border State

Neural induction has often been considered as a choice between neural and epidermal fates in the ectoderm. Evidence mainly from experiments in *Xenopus* led to the influential "default model" (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b), which proposes that no instructive signalling is required for neural fate; rather, when cells in the ectoderm are relieved of inhibitory BMP signalling, they will differentiate towards their default, neural fate. Thus, by such a mechanism ectodermal cells in the blastula stage *Xenopus* animal cap are able to be neuralised by the addition of BMP inhibitors (Lamb et al., 1993; Sasai et al., 1995) or by knocking down BMP signalling effectors (Hawley et al., 1995; Sasai et al., 1995; Xu et al., 1995). Thus, the secretion of BMP antagonists by the organizer (Smith and Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1995) has been suggested to account for events of neural induction by default (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b), a model more recently extended to mammalian embryos (Levine and Brivanlou, 2007). However, this model has been difficult to reconcile with findings in the chick; that no combination of BMP antagonists can induce neural markers in competent area opaca epiblast (Streit et al., 1997; Streit et al., 1998; Streit and Stern, 1999; Streit et al., 2000; Linker and Stern, 2004), also confirmed here (Chapter 3), that the BMP antagonists Noggin and Chordin are not expressed until after the neural plate has formed (Streit and Stern, 1999), and that addition of BMP does not inhibit formation of the neural plate (Streit et al., 1998; Streit and Stern, 1999) other than at the neural plate border (Pera et al., 1999; Streit and Stern, 1999).

Here, an explanation for these differences is proposed by highlighting the previously unappreciated role of neural plate border cell state in cells capable of responding to BMP signals. Thus cells in the chick ectoderm require cell continuity with the border to be neuralised by BMP inhibitors, and *Xenopus* ectodermal animal caps can be neuralised by BMP inhibitors as they contain prospective border cells. Similarly explants from early, pre-streak chick embryos can respond to BMP signals (Wilson et al., 2000) because they enter a border-like cell state in culture. These observations are also consistent with the finding that BMP antagonists only enlarge the neural plate in *Xenopus* microinjection studies, if the constructs are injected into blastomere cells that give rise to border cells (Linker and Stern, 2004; Delaune et al., 2005).

BMP inhibitor misexpression experiments in the ectoderm therefore suggest that there is something qualitatively different about the cells in the border region, as epidermal cells in contact with the neural plate exposed to BMP inhibitors will express neural and border markers; epidermal cells not in contact will express only border markers, but not neural markers. Thus, BMP inhibition can only effect a single step in converting prospective epidermal cells; shifting them one-step to a neural plate border-like state, but not a second step to enable them to express neural markers.

However, it has been suggested (Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000) that the reason why BMP inhibitors are unable to induce neural markers in the chick is that BMP is not fully inhibited by the methods used. These results (and the finding that high concentration of Noggin is unable to induce *Bert* or *Sox2* - Chapter 2) suggest that that the levels of BMP inhibition used are sufficient to effect fate changes in ectodermal cells, and that ultimately the reason why ectoderm cells cannot be neuralised by BMPs is that only cells in contact with the border can respond (Pera et al., 1999; Streit and Stern, 1999) (Chapter 5).

What could be the missing signal in the neural plate, which can only travel between BMP-inhibited cells? Given that cell-cell contact is required for this phenomenon, one of the best candidates is Notch; a cell-cell signal previously

implicated in forming the boundary at the border of the neural plate (Kintner, 1992; Cornell and Eisen, 2002; Endo et al., 2002; Glavic et al., 2004) and the boundary between germ layers during gastrulation in both *Xenopus* (Revinski et al., 2010) and zebrafish (Kikuchi et al., 2004; Revinski et al., 2010). Thus, modulation of BMP at the border could affect Notch activity thereby extending the border outwards.

An alternative perspective is to consider that high levels of BMP at the border of the neural plate may act to confine the spread of a neuralising signal to just the neural territory. Such a relay mechanism between cells with low levels of BMP could help to synchronise the onset of neural commitment in the neural plate, an affect Notch has been reported to have in the initial stage of differentiation of ES cells (Lowell et al., 2006). This relay/synchronisation mechanism could also provide a way for the organizer to induce neural tissue in regions that never come in to close proximity, and are thought to require the additional signals from either nearby tissue such as the AVE (Klingensmith et al., 1999; Knoetgen et al., 1999a; Knoetgen et al., 1999b) or multiple additional tail organizers (Furthauer et al., 2004; Kudoh et al., 2004; Rentzsch et al., 2004).

In summary BMP misexpression studies herein have identified that only the cells of the neural plate border and epidermal cells in continuity with it are able to respond to BMP inhibitors, and identified the importance of the neural border state in understanding why *Xenopus* animal caps and chick epiblast explants can be affected by BMP signals. Furthermore, there is something quantitatively different about the border when compared to BMP inhibited epidermis, as BMP inhibition alone can only modulate fates in a single step: neural <> border and epidermal<>border, but not neural<>epidermal.

Is lens a default fate?

The lens' function is to focus light on to the fovea at the back of the eye. It does this by containing proteins named Crystallins (Wistow and Piatigorsky, 1988), that refract light. This enables light to pass through the lens and on to the retina. Prior to the formation of the lens, the precursors cell that form it are located in the ectoderm that overlies the optic cup in a placode, or plate-like

thickening of the ectoderm. Cells in the lens placode will go on to develop the two structures of the lens, an outer epithelial sheet and inner sheets of lens fibers containing Crystallins. Classical grafting experiments by Spemann (Spemann, 1901a) and Lewis (1904) (Lewis, 1904) had suggested that the optic vesicle (an outgrowth of the neural tube that will give rise the structures the eye) induces the lens to form in the overlying cranial ectoderm. However subsequent experiments have shown that this was due to contamination of the donor-grafted optic vesicles with presumptive lens progenitors (Henry and Grainger, 1987; Grainger et al., 1988; Henry and Grainger, 1990). Current models suggest that a complex multistep process involving multiple signals is involved in the formation of the definitive lens (Baker and Bronner-Fraser, 2001; Chow and Lang, 2001; Litsiou et al., 2005; Schlosser, 2006). It is therefore unexpected that pre-streak, stage-XII epiblast explants should express specific lens markers including *δ-crystallin* after extended culture (6 days).

The rationale for looking for lens markers was because explants cultured for a short time period expressed several markers indicative of the neural plate border, pre-placodal region (including *Dlx5, Gata3, Six4*, *Eya2, Pax6*) (Schlosser, 2006; Streit, 2007), a region of the ectoderm bordering the neural plate that has lens as its ground-state of specification (Bailey et al., 2006), as shown by in vitro culture. Thus the finding that epiblast is also specified as lens supports the finding that it initially passes through a border-like state. Nevertheless, it is remarkable that such cells are able to differentiate from a multipotent state without additional instructive signals towards a lens fate.

How is it possible for epiblast culture to differentiate towards the lens fate without additional, instructive signalling? Initially the state of the pre-streak epiblast is border-like; this is correlated with the expression *ERNI* and *Msx1*, both of which are later expressed at the border of the neural plate (Streit and Stern, 1999; Streit et al., 2000) and with the fact that cells from any region of the epiblast can give rise to cells of the neural plate border (Hatada and Stern, 1994). Thus, one interpretation is that at the time of culture, epiblast cells

already express markers of the border and therefore undergo a developmental trajectory in culture only normally seen in a subset of cells at the border later in development.

However, it is also possible that culture conditions could be responsible for this differentiation. The medium contains insulin, and IGF has been shown to generate anterior neural fates and ectopic structures, including ectopic eyes, when injected into *Xenopus* embryos (Pera et al., 2001). Furthermore, insulin-like growth factor-I has been suggested to regulate *δ-crystallin* expression (Alemany et al., 1989). However, if the expression of *δ-crystallins* is driven directly by IGF it might be expected that cells would respond within 40 hours as well as 6 days, which is not the case.

IGFs are known to signal through the MAPK pathway, like FGF, and whilst FGF has been shown to be required for formation of the PPR (Streit, 2004), maintenance of the lens (Zhao et al., 2008) and lens fibre differentiation (Lovicu and McAvoy, 2005; Robinson, 2006), it has also been suggested that FGFs can have a repressive effect on lens development (Bailey et al., 2006). Thus although IGF signalling could be responsible for supplementing endogenous FGF signalling in the explanted tissue, it cannot be responsible for a continuous, instructional effect. A single signal like FGF is not sufficient to induce lens fate in pluripotent cells.

The expression of genes known to be induced by BMP signals, including *Msx1 (Suzuki et al., 1997; Tribulo et al., 2003), Dlx5 (McLarren et al., 2003), Gata3* (Curchoe et al., 2010) also argues against instructional (as opposed to a permissive) effect from insulin signalling in the medium, as IGF has been reported to inhibit BMP signalling, by interacting with the downstreram effectors of the pathway (Pera et al., 2003). Thus, medium conditions cannot account for the all the signals that can direct cells to a lens fate.

Furthermore, many of the genes expressed in epiblast explants in short-term culture, such as *Sox3, Sox2*, *Pax6* and *Otx2* (Wilson et al., 2000), are

transcription factors involved in lens induction and the expression of lens specific Crystallins (Kamachi et al., 1998; Tanaka et al., 2004; Inoue et al., 2007). For example, *Sox3* has been suggested to confer lens competence in the surface ectoderm (Zygar et al., 1998; Koster et al., 2000), and *Otx2* has been associated with lens bias (Zygar et al., 1998). *Pax6* is highly conserved in eye development (including the lens) throughout evolution, as shown by its misexpression in organisms as divergent as *Drosophila* and *Xenopus*. Misexpression of *Pax6* can cause the formation of ectopic eyes in both these species (Halder et al., 1995; Chow et al., 1999). Moreover, *Sox2* and *Pax6* have been shown to act cooperatively at the enhancer region of *δ-crystallin* and *L-maf,* lens genes (Kondoh et al., 2004) and overexpression of *Sox2* and *Pax6* together (but not individually), in the cranial ectoderm of the chick is sufficient to induce the expression of ectopic placodal thickening that express *δ-crystallin* (Kamachi et al., 2001). Thus, it can be seen that the complement of transcription factors expressed after short-term culture, are key effectors of lens development. One explanation for the finding that epiblast explants can express lens markers in extended culture is that transcription factors are re-used throughout development for different purposes. Genes like *ERNI, Msx1, Sox3, Otx2* all have dynamic expression patterns; *Sox3* is expressed early and maintained in prospective neural tissue (Rex et al., 1997; Streit et al., 2000), *Msx1* and *ERNI* are expressed in the area opaca epiblast then shifted to the border of the neural plate (Streit and Stern, 1999; Streit et al., 2000; Khudyakov and Bronner-Fraser, 2009), and *Otx2* is expressed early in the area opaca epiblast, then later in the anterior neural plate (Bally-Cuif et al., 1995). Thus, by culturing cells from this early time-point whose cells state is similar to that as the border, cells follow a developmental trajectory that enables them ultimately to become lens.

One remaining question is whether this early border-like phase in the pre-streak epiblast is a general cell state of all early epiblast-like cells. To answer this, cell culture experiments on chick ES cells were developed to see if these pluripotent cells whose differentiation could be initiated by medium condition would then go on to differentiate to a lens fate. Recently, a study using human

ES cells has been able to derive lentoid-bodies, expressing several of the human-specific lens Crystallins (αA, aβ, β) over a 35 day protocol (Yang et al., 2010). In this study cells expressed Pax6 after 4 days with FGF4 and Noggin treatment, and formed lentoid bodies after culture with Wnt3a (Yang et al., 2010). Thus, this shows that ES cells can differentiate towards lens like fate with minimal sets of instructive signals. Differences between the study of Yang et al., (2010) and the 9N2 study herein, could be due to difference in addition of Noggin, enabling FGF mediated differentiation to be maintained, and the use of Wnt. In the chick ES study herein, cells appeared to be in a neural crest-like state after retinoic acid treatment, hence given that Wnts induce neural crest (Trainor and Krumlauf, 2002; Li et al., 2009; Steventon et al., 2009) and Wnt inhibition is required for pre-placodal fates (Litsiou et al., 2005; Ohyama et al., 2006) a Wnt inhibitor, Dkk1 was added to cultures. However, it will be interesting to see if the addition of Wnt will derive lentoid cells from chick ES cell culture.

In summary, the ground-state of specification of the early chick epiblast is initially border-like and ultimately lens. The pre-streak chick epiblast expresses border like markers; its fate, like that of the border, can be affected by BMP signalling, and cells from anywhere in the epiblast can contribute to the border. The question of whether all early prospective neural cells share this ground-state of specification with chick epiblast cells remains.

Early Events in Neural Induction

The results herein support an instructive (but not sufficient) role for FGF signalling in neural fate acquisition, supporting previous studies showing the importance of FGF (Streit et al., 2000; Wilson et al., 2000), and those showing that an early phase of FGF is required prior to BMP inhibition (Linker and Stern, 2004; Delaune et al., 2005). FGF is required because it initiates the expression of a cascade of response genes in responding tissue. Of 10 genes up-regulated in response to the first 5 hours of signalling by a grafted node, 8 have been shown to be regulated by FGF (Asterix, *ERNI, Churchill, Calfacilitin, Dad1, polyubiquitin, and ferritin heavy chain*, *Obelix)*(Streit et al.,

2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Pinho et al., 2011), none of which can be induced by BMP antagonists.

One of the earliest events in neural induction in the early epiblast must consist of a transition from a pluripotent state to a pre-neural/border state of prospective neural cells (Chapter 4). Indeed genes known to control plurpotency in stem cells (Boiani and Scholer, 2005; Orkin et al., 2008; Ng and Surani, 2011), including *Oct4* and *Nanog* (Lavial et al., 2007), have been shown to be expressed broadly in the pre-streak epiblast, but later clear from the region surrounding the node from stage 5 (Lavial et al., 2007). This corresponds with the time at which commitment to germ layer fates has been suggested to occur (Garcia-Martinez et al., 1997), as shown by heterotopic germ layer transplantation experiments. Interestingly, the expression of pluripotency genes is maintained in a wide region of the embryo encompassing the neural plate border (Lavial et al., 2007), and by stage 6 the only region of the embryo to express these markers is the most anterior edge of the neural plate (Lavial et al., 2007). This suggests that genes like *ERNI* expressed early in the epiblast, and then later at the border (Streit et al., 2000) of the neural plate might be considered to mark regions of mulitpotency.

These results also show that neural induction does not progress in a single step. Genes are expressed with a temporal hierarchy, which is reflected in both their expression during normal development in prospective neural tissue and in the timing of their induction by a grafted node (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Pinho et al., 2011) (Chapter 2). The question of how a single signal can achieve this temporal regulation remains unknown. One possibility is that initiates a transcriptional network in which interrelated transcription factors regulate and feedback on each other, as has been described for both cell types; for example the neural crest (Sauka-Spengler et al., 2007; Sauka-Spengler and Bronner-Fraser, 2008), and cell states such as pluripotency in embryonic stem cells (Boiani and Scholer, 2005; Orkin et al., 2008; Ng and Surani, 2011). Alternatively, cells could through an unknown mechanism measure the duration of their exposure to a signal. Such an exposure model has been

proposed for controlling the identity of digits in the limb (Towers et al., 2011), where the length of exposure to SHH "promotes" (Towers et al., 2011) cells between different digit identities.

It has been suggested from *Xenopus* experiments that a combination of FGF and BMP inhibition is sufficient for neural induction (Marchal et al., 2009). However, a combination of high levels of BMP and FGF is not sufficient to induce *Sox2* in the chick, nor can a combination of FGF8a and *Smad6* induce neural markers in the ventral ectoderm of *Xenopus*. Furthermore, two neural response genes, *TrkC* and *Bert* (Chapter 3), are not induced by Noggin or a combination of FGF and BMP inhibitors. Thus, signals in addition to these must be required.

A Multi-Step Model of Neural Induction.

In conclusion the results herein supports a multi-step model for neural induction. FGF signalling probably from the hypoblast layer (Stern, 2006; Albazerchi and Stern, 2007), initiates the first steps in the neural induction cascade (*ERNI, Sox3* and *Calfacilitin*) (Streit et al., 2000) (Panpanyotou unpublished observations) in pre-steak epiblast, a cell state, which corresponds to a border-like state (Chapter 5). These cells are still multipotent (Garcia-Martinez et al., 1997), and progenitors of the germ layers are somewhat intermixed (Hatada and Stern, 1994). During this stage *ERNI* acts to prevent precocious *Sox2* expression (Papanayotou et al., 2008) keeping cells in a multipotent state, but priming them for later expression.

Prior to gastrulation FGF8 signalling from the hypoblast layer is augmented by FGF8 expression from Koller's sickle (Streit et al., 2000). As gastrulation starts, FGF8 expression is retained in most of the streak including Hensen's node (which is derived from two cell populations, one of which is situated in Kollers' sickle) (Izpisúa-Belmonte et al., 1993; Streit et al., 2000). At this time, the "second epoch" of genes begin to be expressed including *Asterix, Churchill, Dad1, polyubiquitin, and ferritin heavy chain*, and *Obelix* (Streit et

al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010; Pinho et al., 2011). *Churchill* acts thorough *Sip1 (Sheng et al., 2003)* to inhibit mesodermal fate in prospective neural cells and defines a mesodermal-neural boundary and *Dad1* prevents premature apoptosis of prospective neural cells (whilst together with *polyubiquitin, and ferritin heavy chain* increasing apoptosis at the border) (Streit et al., 2000; Sheng et al., 2003; Papanayotou et al., 2008; Gibson et al., 2010; Papanayotou et al., 2010). From this point onwards BMP inhibition can now play a role in the maintenance of neural fate (Streit et al., 1998) perhaps through a mechanism involving Smad-interacting protein1 (Chapter 4). However, signals in addition to FGF and BMP inhibition secreted by the node are now required to induce the remaining genes in the cascade, including *TrkC*.

Finally, commitment of prospective neural cells to neural plate has been proposed to depend on BERT, which relieves the inhibitory complex containing ERNI from the N2 enhancer of *Sox2* (Papanayotou et al., 2008). *Sox2* is now expressed throughout the neural plate marking the commitment of cells to the neural plate (Gallera and Ivanov, 1964; Gallera, 1970).

Future Directions.

The primary goals of any future study should be to identify what regulates *TrkC* and *Bert* as these provide two useful markers; *TrkC* for the "maintenance phase" of neural induction (Chapter 1), and *Bert* for the onset of neural plate commitment. A combination of bioinformatics and gain- and loss-of-function experiments should allow the signals that regulate these genes to be identified.

Ultimately, the question of why it takes 12 hours to induce *Sox2* in the area opaca and to induce definitive neural plate fate needs to be addressed. It is unknown what genes are expressed between 5 and 12 hours in the neural induction cascade. A differential screen comparing area opaca epiblast that has been exposed to signals from the organizer for 9 hours would be highly useful in identifying the missing genes.

Finally, gastrulation is a highly complex and dynamic process involving multiple signals. Understanding how cells can integrate and respond to the level of signals such as SMAD1 and SMAD2 is important. The development of an assay or combination of approaches, such as BiFCo (Hu et al., 2002; Hu et al., 2006), FRET (Fluorescence Resonance Energy Transfer) (Wu and Brand, 1994; Jares-Erijman and Jovin, 2003) or co-immunoprecipitation (Bonifacino et al., 2001; Masters, 2004), could enable this to be investigated, and enable an understanding of how cells make fate decisions in a dynamic environment.

Conclusion

In conclusion, the work herein supports a model of neural induction involving sequential steps and involving a cascade of genes induced by multiple signals. The early steps of neural induction are coordinated by FGF signals, and signals in addition to FGF and BMP inhibition are required. However, neither retinoic acid, somatostatin, noggin, insulin, or an increase intracellular calcium are sufficient to induce *TrkC* or *Bert*, genes for which no signals are currently known.

Finally, this work highlights a previously unappreciated role of the border-state, which has important implications for understanding BMP misexpression studies at neural plate stages, and in the cell state of the epiblast as culture studies suggest that cells initially enter a state similar to that of the neural plate border, confirmed by their subsequent differentiation into lens. Overall, the experiments reveal a hitherto unknown importance of a neural border cell-state, and suggest that lens is the ground state at the start of the neural induction cascade.

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